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Characterization of *Klebsiella pneumoniae* bacteriocalins

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RESUMO

Desde a descoberta dos primeiros antibióticos em meados do seculo XX, que estes têm vindo a ser utilizados, frequentemente, de forma indiscriminada para combater infeções bacterianas, o que tem sido associado a um aumento do número de infeções causadas por bactérias multirresistentes (MDRs). Apesar de várias medidas terem sido já implementadas para evitar a incidência de microrganismos multirresistentes, estas têm-se mostrado ineficazes, ficando a comunidade científica responsável por encontrar novas formas de combater os mecanismos de resistência bacterianos.

Um dos mecanismos recentemente descritos envolve a actuação de proteínas denominadas lipocalinas bacterianas (BCNs, anteriormente conhecidas por YceI). Inicialmente descritas em *Burkholderia cenocepacia* K56-2, estas proteínas são secretadas pelas bactérias capturando antibióticos hidrofóbicos no meio extracelular, impedindo o antibiótico de atuar sobre a bactéria, aumentando assim a concentração mínima inibitória (CIM) do respetivo antibiótico. Este mecanismo não aparente ser restrito às bactérias produtoras de BCNs, exercendo actividade e proteção sobre a comunidade bacteriana envolvente.

As BCNs consistem em proteínas de baixa massa molecular, altamente conservadas entre as bactérias, apresentando uma conformação característica tridimensional (3D) de barril-*β*, seguida por uma hélice-*α*. Geralmente secretadas para o meio periplasmático, livres ou ancoradas à membrana plasmática, estas proteinas podem ainda surgir no meio extracelular ou, ainda, no citosol da bactéria. As BCNs descritas até à data ainda se encontram muito pouco caracterizadas funcionalmente, desconhecendo-se a capacidade de ligação de proteínas ortologas das descritas originalmente em alguns organismos aos antibioticos. Desta forma, esta tese focou-se no estudo do homólogo de BCN em *Klebsiella pneumoniae* kp52.145 designado BcnK. Esta bactéria Gram-negativa, pertencente à família das *Enterobacteriaceae*, é um importante agente patogénico responsável por surtos de pneumonia, entres outras infeções, em ambientes hospitalares e na comunidade. Tal como se verifica com outros agentes patogénicos, o número de estirpes de *K. pneumoniae* multirresistentes tem vindo a aumentar. Os principais mecanismos de resistência presentes nas estirpes clínicas desta espécie consistem em: (i) produção de β-lactamases de largo espectro de atuação, capazes de hidrolisar cefalosporinas e antibióticos do grupo dos monobactâmicos, ou (ii) produção de carbapenemases, que possuem a capacidade de hidrolisar um largo espectro de antibióticos, incluindo carbapenemes. Estes genes possuem a capacidade de se propagar horizontalmente entre estirpes da mesma ou outras espécies, tendo sido detetadas em várias regiões do mundo, constituindo assim uma ameaça para a saúde pública.

Assim, a fim de se caracterizar funcionalmente a proteína BcnK, o gene correspondente (*bcnK*) foi expresso por clonagem em *Escherichia coli* no vetor pDA-CTHis, contendo uma cauda de seis histidinas na extremidade C-terminal, dando origem a pDG1. A proteína recombinante expressa foi purificada com sucesso por cromatografia de afinidade. A verificação da expressão desta proteína foi efectuada através de SDS-PAGE e Western-blot. Apesar de se ter verificado que a expressão de BcnK conferiu um aumento da CMI, traduzido por uma diferença de

crescimento de cerca de 65.5% OD_{600} a uma concentração de 1.0 µg/mL de polimixina B, em testes de proteção com *Pseudomonas aeruginosa* PAO1, não foi possível repetir este ensaio devido à agregação da proteína observada durante a diálise em tampão PBS, o que impossibilitou a quantificação da proteína e a sua utilização em ensaios subsequentes. Especula-se que a agregação observada se deveu, possivelmente, à componente lipídica N-acil-S-sn-1,2-diacilcerilcisteína presente na exterminade N-terminal de BcnK. Desta forma, procedeu-se a nova tentativa de clonagem de *bcnK* no mesmo vetor, removendo-se a sequência do péptido sinal. Contudo, não se obtiveram quantidades suficientes da proteína produzida para se proceder à sua purificação. Assim, alternativamente, clonou-se *bcnK* no vetor induzível por IPTG, pET-28a (+), contendo caudas de histidina nas extremidades N- e C- terminal (pDG7) e, em paralelo, clonou-se no mesmo vetor, usando apenas uma cauda de histidinas na extremidade N-terminal (pDG8). A expressão do gene *bcnK* nestes vetores levou à produção de quantidades suficientes de proteína para purificação. No entanto, a expressão da construção genética em pDG7 conduziu a elevados níveis de agregação da proteína durante a diálise, possivelmente devido à presença das caudas de histidina. Contrariamente ao esperado, a expressão de BcnK em pDG8 não conduziu ao aumento da CMI nos ensaios de proteção. Desta forma, admitiu-se a hipótese de que a proteína recombinante produzida sem péptido sinal teria uma conformação incorreta para o exercício da sua função biológica ou de que a cauda de histidinas presente na extremidade N-terminal pudesse gerar interferência com a atividade da proteína. Um novo plasmídeo foi construído utilizando o vetor pUC19 contendo uma caude de histidinas na extremidade C-terminal. Contudo, a expressão neste plasmídeo também não produziu quantidades suficientes de proteína para purificação. Uma nova abordagem será realizada ao clonar bcnK num vector contendo um péptido sinal, secretando BcnK para o espaço periplásmico sem a componente lipidica N-acil-S-sn-1,2-diacilcerilcisteína, permitindo a solubilização da proteína.

De forma a investigar o papel de BcnK na resistência a antibióticos exibida por *K. pneumoniae*, tentou-se inativar *bcnK* no genoma bacteriano por duas metodologias, através de mutagénese dirigida não marcada, o que permitiria a obtanção de um mutante de eliminação isogénico, e através de mutação por inserção, por recurso aos plasmídeos construídos neste trabalho, pDG2 e pDG9, respetivamente. No entanto, não foram obtidos mutantes por quaiquer dos métodos, tendo-se obtido mutantes merodiplóides apenas na estratégia de inativação por mutagénese dirigida não marcada. Assim, em alternativa, procurou-se testar a essencialidade de BcnK por expressão de *bcnK* sob o controlo de um promotor induzível por ramnose. Contudo, foi necessário testar a funcionalidade deste promotor em *K. pneumoniae*. Para o efeito, utilizou-se o vetor pSCrhaB2-e-GFP, que tem um promotor induzível por ramnose fundido transcricionalmente com o gene GFP (*green fluorescent protein*), o qual permite a deteção da sua expressão por fluorescência. Conjugou-se em *K. pneumoniae*, a qual foi crescida em 0.2% e 0.5% de ramnose e 0.5% glucose, respetivamente, tendo-se registado fluorescência na presença das diferentes concentrações de ramnose e, por outro lado, a ausência de fluorescência na presença de glucose, o que sugere o correto funcionamento do promotor no hospedeiro *K. pneumoniae*. Um fragmento de *bcnK* foi então clonado no vetor suicida contendo

um promotor induzível por ramnose pSC200, dando origem a pDG10, sendo este posteriormente conjugado em *K. pneumoniae*. Os transconjugantes obtidos foram crescidos em meio M9 contendo 0.5% de ramnose (condições permissivas) ou glucose (condições não permissivas). Em ambos os meios, observou-se crescimento, sugerindo que *bcnK* não é um gene essencial à viabilidade de *K. pneumoniae*, pensando-se ainda que a expressão de *bcnK* na presença de glucose, ainda que em níveis basais, poderá ser suficiente para suportar a viabilidade de *K. pneumoniae*. Informação recolhida durante a pesquisa bibliografica sugere que BcnK intervem ao nivel da cadeia de transporte eletrónico, sendo a expressão de BcnK suprimida em anaerobiose. Desta forma, numa tentativa de se desligar a cadeia de transporte eletrónico, tentou-se mutagenizar *bcnK* em condições de anaerobiose.

Alternativamente, testou-se o papel de BcnK na resposta da célula ao stress oxidativo, usando para o efeito um plasmídeo com um gene repórter que codifica para uma proteína luminescente, a luciferase. Os resultados preliminares mostram o aumento da expressão de P_{bcnK}*::luxCDABE* e P_{oxyR}*::luxCDABE* (controlo positivo) nas condições testadas, sem grandes diferenças aparentes. No entanto, pretende-se utilizar no futuro a construção P_{waaE}*::luxCDABE* como controlo negativo, pois é previsto que a expressão de *waaE* não seja induzida por stress oxidativo.

O mutante de *B. cenocepacia ΔBcnAΔBcnB* foi complementado com o plasmídeo pDG1 expressando BcnK para verificação da possibilidade de recuperação do fenótipo em relação à estirpe selvagem. Verificou-se que a complementação com BcnK conduz a níveis de CMI similares aos verificados com a estirpe selvagem, no entanto o mesmo resultado foi obtido quando se introduziu apenas o vetor (controlo), sugerindo que um efeito inespecífico. No entanto, *bcnK* será clonado no vetor pSCrhaB2, vetor este utilizado originalmente nos estudos de complementação em *B. cenocepacia*.

O alinhamento das sequências aminoacídicas de BcnK e BcnA por recurso à ferramenta Clustal Omega demonstrou que os resíduos Val107 e Glu118 de BcnK parecem corresponder aos resíduos Asp82 e Asp93 de BcnA, respetivamente, que foram demonstrados como essenciais para a ligação de BcnA a antibióticos. Apesar destas evidências *in silico*, a intervenção dos resíduos correspondentes em BcnK ao nível da ligação com antibióticos permanece por demonstrar experimentalmente.

A análise de polimorfismos de BcnK por pesquisa de homologia nas bases de dados internacionais, restringindo a busca ao género *Klebsiella*, seguida pelo alinhamento das respetivas sequências aminoacídicas, demonstrou que BcnK é altamente conservada neste género (99 a 81% de homologia), contudo desconhece-se ainda a influência das diferenças registadas na função biológica exercida pela proteína a nível celular.

O estudo da conservação de genes vizinhos de *bcnK* por análise de sintenia foi realizado a partir da ferramenta SyntTax. Os resultados obtidos permitiram observar a conservação do *locus* genético deste gene entre as várias espécies testadas. Em algumas espécies, o gene de BCNs encontra-se associado ao gene que codifica para o citocromo b561 (CybB). Contudo, não foi encontrada sintenia para *Enterococcus faecium, Streptococcus pyogenes* e *Acetobacterium*

woodii, nos quais se registou a ausência de BCNs e CybB. Estas espécies bacterianas são conhecidas por não possuírem cadeia de transporte eletrónico funcional e por serem anaeróbios restritos. Assim, considerando a globalidade dos resultados obtidos, propõe-se um modelo da funcionalidade das BCNs, sugerindo-se que estas proteínas participam no transporte ou sequestro de compostos hidrofóbicos, tais como quinonas isoprenóides ou vitamina E, especificamente no meio extracelular, por ação de BcnA, ou no meio periplásmico, por ação de BcnB ou BcnK. Estes compostos hidrofóbicos são transportados até CybB, onde são reduzidos, e posteriormente transportados para o meio extracelular e/ou periplásmico, atuando como agentes antioxidantes.

Fica, no entanto, por demonstrar experimentalmente este papel.

Palavras-chave: BCNs; YceI; *Klebsiella pneumoniae*; kp52.145; Proteína sequestradora

ABSTRACT

Antibiotic resistant bacteria have become one of the greatest threats to modern society, especially those bacteria that resist multiple antibiotics (referred to as multidrug resistant; MDRs). Although most well known resistance mechanisms operate within bacterial cells, recent evidence suggests extracellular mechanisms. One of such mechanisms involves bacterial lipocalins (BCNs), which are secreted proteins that capture hydrophobic antibiotics in the extracellular space. BCNs are widely distributed in bacteria. *Klebsiella pneumoniae*, a Gram-negative enteric bacterium, possesses a BCN ortholog. *Klebsiella* species cause hospital and community-acquired infections and antibiotic resistance is in part due to the spread of β-lactamases. In this thesis, I cloned, expressed and purified a *K. pneumoniae* kp52.145 BCN (BcnK). Recombinant BcnK proteins were employed in antibiotic protection assays using *Pseudomonas aeruginosa* PAO1 against polymyxin B (PmB) as a model system. Full-length recombinant BcnK was unstable and formed aggregates that complicated its quantification. However, this protein caused an increase of 65.5% in the OD_{600} of *P. aeruginosa* in the presence of sublethal amount of PmB. Other *bcnK* constructs were made, but either lacked activity or could not be purified. A *bcnK* chromosomal deletion was attempted using protocol to proceed unmarked deletion and another one to mutate by inserting a polar gene cassette. No mutants were obtained in both cases. *K. pneumoniae* kp52.145 *bcnK* gene expression was placed under control of a rhamnose-inducible promoter, but the resulting constructs did exhibit the expected growth defect, showing the same growth phenotype irrespective of the presence of rhamnose (permissive condition) or glucose (non-permissive condition), suggesting that *bcnK* is not essential for *K. pneumoniae* viability. I also investigated the regulation of *bcnK* gene expression. Preliminary results suggest that *bcnK* expression is upregulated under different concentrations of paraquat, a compound that stimulates the production of oxygen radicals. Recombinant *bcnK* was used to complement a *ΔbcnAΔbcnB Burkholderia cenocepacia* mutant by assessing the restoration of rifampicin resistance to parental levels. However, increased resistance could only be attributed to the plasmid vector control but not to the plasmid expressing BcnK. Alignments done using amino acid sequence for BcnK and BcnA from *B. cenocepacia* J2315 showed two residues, Val107 and Glu118 of BcnK to correspond to Asp82 and Asp93 of BcnA, respectively. Previous reports have shown that these residues, in BcnA, are these residues were shown to be crucial for antibiotic binding. BCN genomic studies showed a highly conserved protein (99 to 81% homology) among *Klebsiella* species. Synteny and BLASTp results showed that in some species BCNs are associated with a cytochrome b561 (*cybB*) gene. However, both BCNs and *cybB* genes are absent in strict anaerobes. I suggest a model of BCNs cellular function that involves the hijacking of hydrophobic compounds, such as isoprenoid quinones, and their transport to the membrane where these compounds are reduced and further transported in the extracellular and/or periplasmic space acting as antioxidants.

Keywords: BCNs; YceI; *Klebsiella pneumoniae*; kp52.145; Antibiotic resistance; hijacker protein

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MDRs – Multidrug resistant
MIC - Minimum inhibitory concentration
AmpC – Ampicillin hydrolysing
CTX-M – Cefotaximase
IMP – Imipenemase
MRSA – Methicillin resistant *Staphylococcus aureus*
NDM – New Delhi metallo-β-lactamase
PRSA – Penicillin resistant *Staphylococcus aureus*
VRE – Vancomycin resistant *Enterococci*
VRSA – Vancomycin resistant *Staphylococcus aureus*
VIM – Verona integron encoded metallo β-lactamase
BCNs – Bacteriocalins
CF – Cystic fibrosis
Bcc – *Burkholderia cepacia* complex
PmB - Polymyxin B
RpoS – S sigma factor
PAMPs – Pathogen-associated molecular patterns
ICUs – Intensive care units
ESKAPE – *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudominas aeruginosa* and *Enterobacter* species
rRNA – Ribosomal RNA
LPS – Lipopolysaccharides
HV – Hypervirulent
ESBLs – Extended-spectrum β-lactamases
KPCs – *K. pneumoniae* carbapenemases
MBL – Metalle-β-lactamases
CRE – Carbapenem-resistant *Enterobacteriaceae*
MLST – Multilocus sequence typing
LB – Lysogeny broth
PCR – Polymerase chain reaction
cPCR – Colony-PCR
NCBI – National Center for Biotechnology Information
BLASTp – Basic Local Alignment Search Tool for proteins
His-tag – Hexahistidine tag
IPTG – Isopropyl thiol-β-D-galactoside
PBS – Phosphate-buffered saline
TBS – Tris-buffered saline
WT – Wild type

CDD – Conserved Domain Database

STRING – Search Tool for the Retrieval of Interacting Genes/Proteins

3D – Tridimensional

Phyre2 – Protein Homology/analogy Recognition Engine

SD – Shine-Dalgarno

IBs – Inclusion bodies

GFP – Green fluorescent protein

IQ – Isoprenoid quinone

ETC – Electron-transport chain

ATP – Adenosine triphosphate

ROS – Reactive oxygen species

FNR – Fumarate and Nitrate reductase

sRNA – Small RNAs

Na_2HPO_4 – Disodium phosphate

NaCl – Sodium chloride

NH_4Cl – Ammonium chloride

$CaCl_2$ – Calcium chloride

$MgSO_4$ – Magnesium sulfate

Mg^{2+} - Magnesium

Ca^{2+} - Calcium

DAP – Diaminopimelic acid

Ni^{2+} - Nickel

CO_2 – Carbon dioxide

H_2 - Hydrogen

N_2 – Nitrogen

mM – Millimolar

µM – Micromolar

µg/mL – Micrograms per milliliter

mg/L – Milligrams per liter

w/v – weigh per volume

˚C – Celsius

s – Seconds

min – Minutes

h – Hour

mL – Milliliter

µL – Microliter

rpm – Rotations per minute

g – Gravity

ksi – Kilopound per square inch

GPa - GigaPascal

µm - Micrometer

V - Volt

mV - Millivolt

A – Ampere

bp – Base pairs

OD_{600} – Optical density at 600 nm

nm – Nanometer

%OD_{600} – Percentage of OD_{600}

RLU/OD_{600} – Relative luminescence unites per OD_{600}

kDa - Kilodalton

% - Percent

CHAPTER I – INTRODUCTION

1.1. The Antibiotic Crisis

Since the discovery of penicillin, the first known antibiotic, by Alexander Fleming in 1928, mankind has relied on this and other antibacterial molecules for the treatment of bacterial infections. The antibiotics are molecules used in the treatment and prevention of infections caused by bacteria. However, the "antibiotic era" might come to an end as the majority of the clinically available antibiotics are becoming useless to treat bacterial infections (Figure 1.1) caused by multidrug resistant (MDRs) Gram-positive and Gram-negative strains (Llor & Cots, 2009). This scenario, named the "post-antibiotic era" is considered one of the greatest threats for mankind (WHO, 2014; WEF, 2015). Despite the investment and incentives to research to identify new antimicrobial molecules in the last years (Piddock, 2012), there are considerable challenges to bring them to the market resulting on a long and fastidious process (Nathan, 2004; Wright, 2015). Aggravating this situation, the interest of pharmaceutical companies to search for new molecules has decreased in the last years, resulting in only a few newly approved and reliable drugs (Spellberg, 2011).

Antibiotic molecules inhibit the growth or kill bacteria with minimum impact on the human body. They have different mechanisms of action for which they are categorized in different classes. For instance, (i) β-lactams interfere with cell wall synthesis, (ii) macrolides inhibit protein synthesis, (iii) fluoroquinolones interfere with nucleic acids synthesis, (iv) trimethoprim inhibits metabolic pathways and (v) polymyxins disrupt bacterial membrane. In some cases, bacteria are capable of overcoming this toxicity through several mechanisms which give rise to resistant strains. The antibiotic therapy represents a stressful environment for a sensitive bacterial community, and resistant subpopulations are selected and ultimately able to prevail and proliferate in the community giving rise to one or more resistant strains. In clinical microbiology, a strain is defined as resistant, susceptible or intermediate by comparing the minimum inhibitory concentration (MIC). This is the minimal antibiotic concentration at which bacterial growth is inhibited under standardized conditions *in vitro* (Turnidge & Paterson, 2007), with the predefined susceptibility "breakpoint" of the tested species.

In the past, the bulk of research efforts in antibiotic resistance focused on bacterial cellular functions associated with decreased susceptibility. These included (i) modification of the antibiotics target due to chromosomal mutations, making it unrecognizable to the antibiotic, (ii) production of enzymes that breakdown or modify antibiotic molecules inactivating them, (iii) extrachromosomal elements from other bacteria, such as plasmids, transposons and integrons, which can be accumulated on a single or several strains expressing proteins that inactivate the antibiotic affect, (iv) efflux pumps responsible for expelling several types of antibiotics from inside the cell (v) and decreasing membrane permeability to antibiotics reducing the access to their targets (Levy & Marshall 2004; Tenover, 2006; Alekshun *et al*., 2007). DNA analysis of human bacterial microbiota revealed identical genes harbored by major bacterial pathogens (Sommer *et al*., 2009) and similar genes responsible for the present modern antibiotic resistance were found in the environment and in samples dating back millions of years (Forsberg *et al*., 2012; Bhullar *et*

al., 2012) suggesting a great adaptation capacity to resist the action of antibiotics whose existence predates their use in clinical therapies (Iredell *et al*., 2016) (Figure 1.1).

Compounding the above-described mechanisms of resistance, other factors not related to bacteria adaptation may influence the rise of resistance. Indeed, the necessary MIC to be achieved on an infected patient, regardless of the drug dosing (pharmacokinetics), may not always be attained in the patient (Andersson & Hughes, 2014), especially at the site of infection, since antibiotics do not readily diffuse inside inflamed tissues. Also, the MIC depends on the population density at the site of infection (inoculum effect), as bacteria produce antibiotic resistance proteins that concentrate locally, destroying more antibiotic than an individual cell (Martínez *et al*., 2015). Thus, bacteria may be exposed to sub-lethal antibiotic concentrations, promoting the rise and selection of resistance.

To reduce the incidence of MDRs, new strategies of drug administration are being implemented in hospital facilities (Baquero *et al*., 2011; Spellberg *et al*., 2013). However other measures must also be applied to increase the efficiency of old and currently used drugs. So it falls on scientific community to research for new antimicrobial drugs or augment currently licensed antibacterial drugs (Piddock, 2012) and generate a deep understanding of biological and molecular mechanisms of antibiotic action and resistance (Wright, 2015).

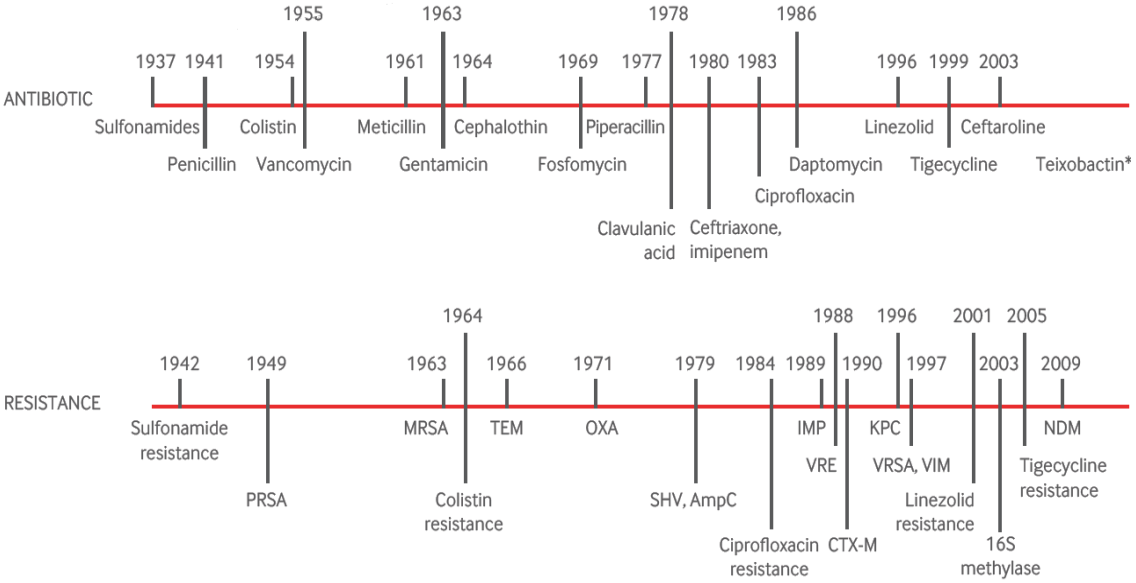

Figure 1.1 – Timelines of antibiotic introduction (above) and bacterial antibiotic resistance (below). *Not in clinical use; Ampicillin hydrolyzing (AmpC); Cefotaximase (CTX-M); Imipenemase (IMP); *Klebsiella pneumoniae* carbapenemase (KPC); Methicillin resistant *Staphylococcus aureus* (MRSA); New Delhi metallo-β-lactamase (NDM); Penicillin resistant *S. aureus* (PRSA); β-lactam hydrolyzing enzymes (TEM, SHV, OXA); Vancomycin resistant *Enterococci* (VRE); Vancomycin resistant *S. aureus* (VRSA); Verona integron encoded metallo β-lactamase (VIM) (Iredell *et al*., 2016).

1.2. A new resistance mechanism

1.2.1. Extracellular antibiotic hijacking

Recent work in Valvano's laboratory has identified a group of previous described proteins known as bacterial lipocalins, herein bacteriocalins (BCNs; formerly known as YceI), which possess the ability to bind and hijack antibiotics in the extracellular space rendering them ineffective, thus augmenting the MIC value. The initial study was carried out using *Burkholderia cenocepacia* K56-2, an opportunistic pathogen responsible for causing chronic infection on immunocompromised patients (O'Neil *et al*., 1986; Poe *et al*., 1977), especially in those with cystic fibrosis (CF) (Isles *et al*., 1984). *B. cenocepacia* K56-2 belongs to *B. cepacia* complex (Bcc), a group of 20 closely related species, phenotypically similar but genetically discrete (De Smet *et al*., 2015) of motile, aerobic, rod-shaped, non-spore forming Gram-negative β-Proteobacteria. In that study, data based on the phenotypes of *B. cenocepacia* wild-type and two mutants defective in the production of BCN paralogues, BcnA (BCAL3311) and BcnB (BCAL3310) (El-Halfawy & Valvano, unpublished data) indicated that these proteins, and in particular BcnA, not only hijack antibiotics but their function could be inhibited by vitamin E.

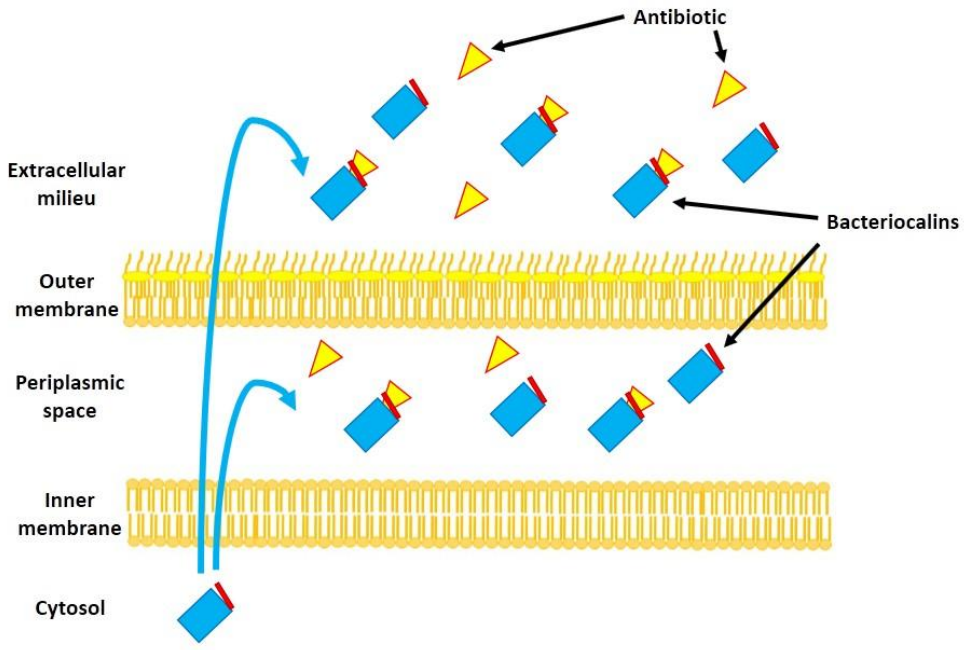

Figure 1.2 – BCNs antibiotic resistance action model. Depending on the homologue, BCN is shown in different cellular localizations, bound to antibiotics.

BCNs are proteins synthesized and secreted into bacterial milieu leading to increased MIC but also virulence augmentation. The described mechanism of action consists on binding to antibiotics rendering them ineffective. This extracellular scavenging of antibiotics by BCNs represents a novel mechanism (Figure 1.2) of intrinsic bacterial resistance. Moreover, as some of these proteins homologous are naturally secreted into the extracellular milieu, its effect is not restricted to BCNs producing bacteria. Other more susceptible bacteria present on the milieu, of the same or different species, can benefit from this protection and thus, this mechanism of

resistance can act at the community level (El-Halfawy & Valvano, 2012; 2013). The same authors also demonstrated that the addition of purified *B. cenocepacia* BCNs is also capable to increasing the virulence of other bacteria species in *in vivo* assays with *Galleria mellonella*. Further, BCNs were also capable to protect *Pseudomonas aeruginosa*, when injected in mice, against Polymyxin B (PmB) action. It was also observed that BCNs expression is upregulated in response to antibiotic oxidative stress conditions (El-Halawy & Valvano, 2013).

1.2.2. Bacteriocalins (BCNs)

BCNs consist on a large family of low-molecular weight proteins with more than 5400 homologous distributed in both Gram-negative and positive bacteria (Bishop, 2000; Smart, 2016). Most members of this family are annotated as "*conserved hypothetical proteins*" referred to as YceI. Apart from a few examples of cytoplasmic BCNs from *Campylobacter jejuni* and *Chlorobium tepidum*, most of BCN coding regions possess a type-1 or type-2 signal peptide, suggesting these proteins are secreted into the extracellular space in Gram-positives and into the periplasmic space in Gram-negative bacteria, or they are covalently modified on the N-terminal with a N-acyl-S-sn-1,2-diacylglycerylcysteine moiety, enabling the protein to anchor on the inner leaflet of the outer membrane as lipoproteins (Bishop, 2000) (Figure 1.3).

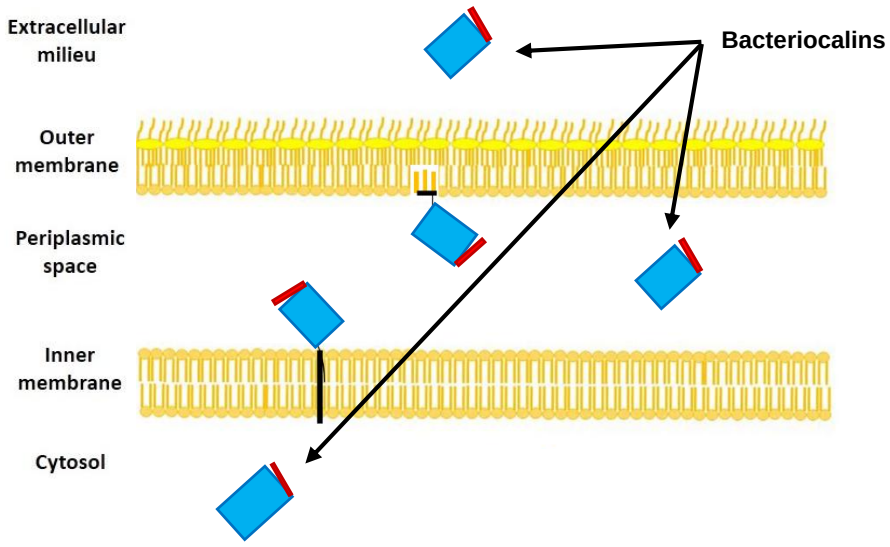

Figure 1.3 – BCNs subcellular localization in bacteria, showing BCNs in cytosol, anchored on inner and outer membrane, in periplasmic space. Its localization depends on the presence or absence and type of leader peptide present on BCN coding region (Image adapted from Bishop, 2000).

The BCN's expression in *Escherichia coli* was shown to depend on S sigma factor (RpoS), which activates gene transcription under several environmental stressful conditions, such as starvation, osmotic stress (Bishop, 2000) and alkaline pH (Stancik *et al.*, 2002). Other example of induced expression occurs in the homologous *Helicobacter pylori* BCN, which is overexpressed under low pH contributing for bacterial survival during a stomachic infection (Sisinni *et al*., 2010).

There are also indications that BCNs homologs from different bacteria possess different functionalities based on the capacity to bind to different molecules, such as fatty acids and amines (Sisinni *et al*., 2010), isoprenoid lipids (Handa *et al*., 2005; Vincent *et al*., 2010), chlorophenoxy herbicides (Benndorf *et al*., 2004), lipophilic antibiotics (El-Halfawy & Valvano, 2013), and fat-soluble vitamins such as α-tocopherol (vitamin E) and menaquinone (vitamin K2) (El-Halfawy & Valvano, unpublished).

The BCNs three-dimensional fold generally consists of an extended, eight to nine stranded antiparallel β-sheet, folding back on itself forming a β-barrel with one closed end, establishing a pocket inside and followed by a C-terminal α-helix (Figure 1.4). The lipophilic ligands are hosted inside the pocket of the β-barrel structure (Bishop, 2000; Handa *et al*., 2005). However, BCNs molecular modeling suggests two binding ways: (i) one by antibiotic polar interactions, with several amino acids residues, at the rim of the BCNs pocket, and another (ii) for more lipophilic interactions deeper into its pocket. Aromatic moieties may also play a role in molecular recognition of these proteins (El-Halfawy & Valvano, unpublished).

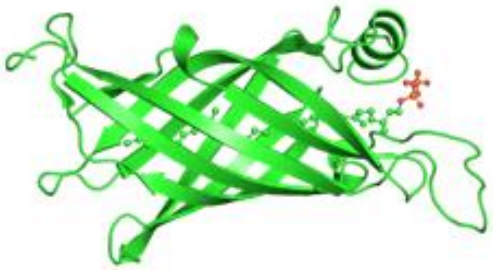

Figure 1.4 – Polyisopreniod-binding protein TT1927b (protein data bank code 1wub), an example of YceI-like crystal structure with a polyisoprenoid within the β-barrel structure, obtained from *Thermus thermophiles* HB8. (http://www.ebi.ac.uk/pdbe/entry/pdb/1wub).

Like many other components present on the bacterial envelope (BCNs are uniquely synthesized by bacteria and are cell surface-exposed), BCNs are recognized as pathogen-associated molecular patterns (PAMPs). The N-acyl-S-sn-1,2-diacylcerylcysteine modification on BCNs N-terminus stimulates an immune response through CD14 receptors recognition in macrophages plasmatic membrane surface (Medzhitov & Janeway, 1997; Hoffmann *et al*., 1999), resulting in activation of immune cells leading to inflammatory responses (Brightbill *et al*., 1999; Aliprantis *et al*., 1999). BCNs stimulate innate and adaptive immune systems, as also indicated by the presence of BCNs antibodies in patients' sera (Scott *et al*., 2013; Yoder-Himes *et al*., 2010; Upritchard *et al*., 2008).

BCNs cellular function is still unclear. Even with all the available information, is not possible to predict the binding preference of the BCN orthologs. In this study, we focused on the *Klebsiella pneumoniae* BCN homolog. *K. pneumoniae* is an opportunistic pathogen responsible for more than 15% of Gram-negative infections in hospital intensive care units (ICUs) in the United States (Lockhart *et al*., 2007), and the main cause of nosocomial infections caused by the *Enterobacteriaceae* in hospitals (Chien Ko *et al*., 2002; Sanchez *et al*., 2013) and community centers (Carpenter, 1990). Rice (2008) referred *K. pneumoniae* as one of the ESKAPE bugs, along with *Enterococcus faecium*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species. These bacteria are responsible for the largest share of nosocomial infections as well representing paradigms of pathogenesis, transmission and, most importantly, antibiotic resistance (Rice, 2008).

1.3. A rising multidrug resistance bacterium

1.3.1. The *Klebsiella* genus

The *Klebsiella* genus includes nonmotile, capsule forming, rod shaped Gram-negative bacteria of the *Enterobacteriaceae* family and belongs to the γ-Proteobacteria. They were named after the German microbiologist Edwin Klebs. This bacterium is human commensal normally found among the skin and gastrointestinal tract microflora (Bagley, 1985). It can also be found in sewage, polluted waters, soil and plants. Some strains associated with plant roots have shown to fix nitrogen, converting into ammonia (Postgate, 1998) through the expression of the nitrogenase enzyme complex, encoded by the *nif* gene cluster (Ausubel *et al*., 1983; Wang *et al*., 2013). Consequently, those strains have the potential to be used for agriculture (Riggs *et al*., 2001; Temme *et al*., 2012). Through genetic comparison of 16S rRNA and *rpoB* gene sequences, *Klebsiella* genus can be organized in three distinct clusters. Cluster I: *K. pneumoniae* containing three sub-species; *K. pneumoniae* subsp. *pneumoniae*; *K. pneumoniae* subsp *rhinoscleromatis* and *K. pneumoniae* subsp. *ozaenea*. Cluster II containing *K. ornithinolytica, K. planticola*, *K. trevisanii* and *K. terrigena*. And cluster III containing *K. oxytoca* (Drancourt *et al*., 2001). *Klebsiella* species are characteristic for producing a thick polysaccharide capsule (Figure 1.5), giving their colonies a mucoid appearance. The capsule synthesis represents a key element to *Klebsiella*'s virulence, as capsulated strains exhibits higher virulence than capsule defective mutants (Simoons-Smit *et al*., 1986). The capsules are generally composed by polysaccharides in repeating units of three to six sugars and uronic acids giving rise to 77 varieties (Li *et al*., 2014; Follador *et al*., 2016), known as K-antigens, enabling to distinguish *Klebsiella* strains into serotypes (Podschun & Ullmann, 1998). Within these K-antigens varieties, strains harboring K1 and K2 capsules types exhibit hypermucoviscous phenotypes due to the elevated production of capsule and are associated with hypervirulence of *Klebsiella pneumoniae* strains (Follador *et al*., 2016), although not all of K1 and K2 strains are virulent (Kauffmann, 1949; Mizuta *et al*., 1983). *Klebsiella* polysaccharide capsule prevents bacteria from being recognized by innate immune defenses, avoiding phagocytosis and inhibiting the complement activation (Domenico *et al*., 1994). The polysaccharides also inhibit differentiation and functional capacity of macrophages in *in vitro* assays (Yokochi *et al*., 1979). The O-antigen, the outermost part of lipopolysaccharides (LPS), represent another virulence factor of the *Klebsiella* genus, as it activates the complement system, causing C3b molecule deposition far from the bacterial cell membrane, thus preventing the insertion of the complement's

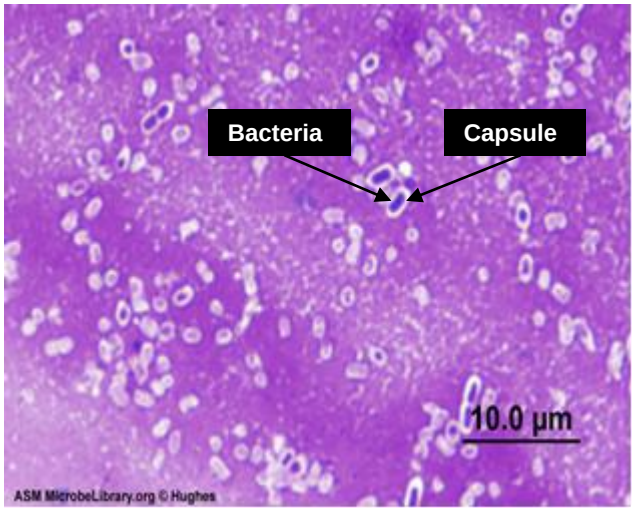

Figure 1.5 - *Klebsiella pneumoniae* (dark violet) surrounded by its capsule (white) grown in skim milk broth and stained with Anthony's capsule stain. (Roxana B. Hughes, University of North Texas, Denton, TX)

membrane attack complex into the bacteria cell envelope (Podshun & Ullmann, 1998). Additional virulence factors in *Klebsiella* include: (i) adhesins (pili, fimbriae) allowing the bacteria to bind to the host cell and catheters (Schroll *et al*., 2010), (ii) siderophores, which are secreted low-molecular-weight, high-affinity iron chelators scavenging iron bound to host proteins (Podshun & Ullmann, 1998), (iii) biofilm formation, which provides increased antibiotic resistance, and (iv) urease production, which, by hydrolyzing urea increases the pH in the infection locale, leading to precipitation and encrustation of organic salts and facilitating the formation of biofilms (Clegg & Murphy, 2016). However, there is a significant heterogeneity in *Klebsiella* strains and not every one of these factors plays the same critical role in virulent strains (Paczosa & Mecsas, 2016). The virulence factors used by *Klebsiella* are not focus in this work, so the reader is referred to recent reviews for additional information (Li *et al*., 2014; Paczosa & Mecsas, 2016).

1.3.2. *Klebsiella pneumoniae*

1.3.2.1. Epidemiology

K. pneumoniae is the most prevalent species of nosocomial agents that cause pneumonia, followed by *Klebsiella oxitoca* (Jarvis *et al*., 1985). *Klebsiella* can rapidly spread in healthcare facilities by direct contact with infected personal or other patients and cause hospital outbreaks (ECDC, 2014) and community-acquired pneumonias (Paczosa & Mecsas, 2016). Apart from pneumonia, *K. pneumoniae* is also frequently responsible for urinary tract infections, being the second most frequent cause after *E. coli* (Czaja *et al*., 2007; Lorente *et al*., 2005). *K. pneumoniae* primary infections on lungs and bladder can spread into the blood causing bacteremia, resulting in high rates of morbidity and mortality (Clegg & Murphy, 2016). Individuals suffering from *diabetes mellitus*, neuropathic bladders, chronic renal insufficiency, chronic obstructive pulmonary diseases, immunocompromised patients and alcoholics are generally considered risk groups for *K. pneumoniae* infections (Montgomerie, 1979; Ko *et al*., 2002; Clegg & Murphy, 2016), although *K. pneumoniae* hypervirulent (HV) strains were previously reported to cause life-threatening infections on healthy individuals (Shon & Russo, 2012; Shon *et al*., 2013). Liver infections are likely initiated from a breach in host defenses in the gastrointestinal tract (Paczosa & Mecsas, 2016). These type of strains are primarily responsible of causing liver abscess and are able to spread through metastasis causing further complications such as meningitis, endophthalmitis, necrotizing fasciitis and abscesses in other tissues (Siu *et al*., 2012) and its prevalence seems to be restricted mainly in Taiwan and Southeast Asia (Chung *et al*., 2007; Siu *et al*., 2012). Infections caused by hypervirulent strains are treatable through very aggressive therapies, although these may not prevent catastrophic disabilities on infected patients (Fang *et al*., 2000).

1.3.2.2. *K. pneumoniae* MDRs overview

Much like other important pathogens, *K. pneumoniae* MDRs strains have emerged due to several mechanisms such as efflux pumps (Ogawa *et al*., 2005), chromosomal mutations and plasmid-borne resistance (George *et al*., 1995; Hudson *et al*., 2014). Two major types of resistance have been commonly observed in *K. pneumoniae*. One is the expression of extended-spectrum β-lactamases (ESBLs) capable to hydrolyze cephalosporins and monobactams. The other mechanism which is considered more concerning is the expression of carbapenemases. These are β-lactamases with the ability to hydrolyze carbapenems. First discovered in 1996 (Yigit *et al*., 2001), the *K. pneumoniae* carbapenemases-producing strains (KPCs) render useless a broad spectrum of antibiotics like penicillins, all cephalosporins, monobactams, β-lactamases inhibitors and last-resort carbapenems (Papp-Wallace *et al*., 2010). The functional Ambler classification divides β-lactamases in four major classes (class A to D) based upon their amino acid sequence homology (Hall & Barlow, 2005). β-lactamases from classes A, C and D have serine in their active site, while class B possesses zinc (Hall & Barlow, 2005; Paterson, 2006). In this classification, carbapenemases are included on classes A, B and D, while ESBLs are strict to class A (Queenan & Bush, 2007). All KPCs fall into class A and are divided 16 different variants (KPC-2 to KPC-17), being KPC-2 and KPC-3 the most prevalent (Hirsch & Tam, 2010; Wang *et al*., 2014). Additional types of carbapenemase were also found in *K. pneumoniae* strains such as metallo-β-lactamases (MBL), which fall into Class B and OXA-β-lactamases, which fall into class D (Pitout *et al*., 2015). Irrespective of the type of carbapenemases they encode carbapenemase-producing isolates are usually termed carbapenem-resistant *Enterobacteriaceae* (CRE) (Paczosa & Mecsas, 2016). Only few antibiotics, such as tigecycline and polymyxins, can be used to treat

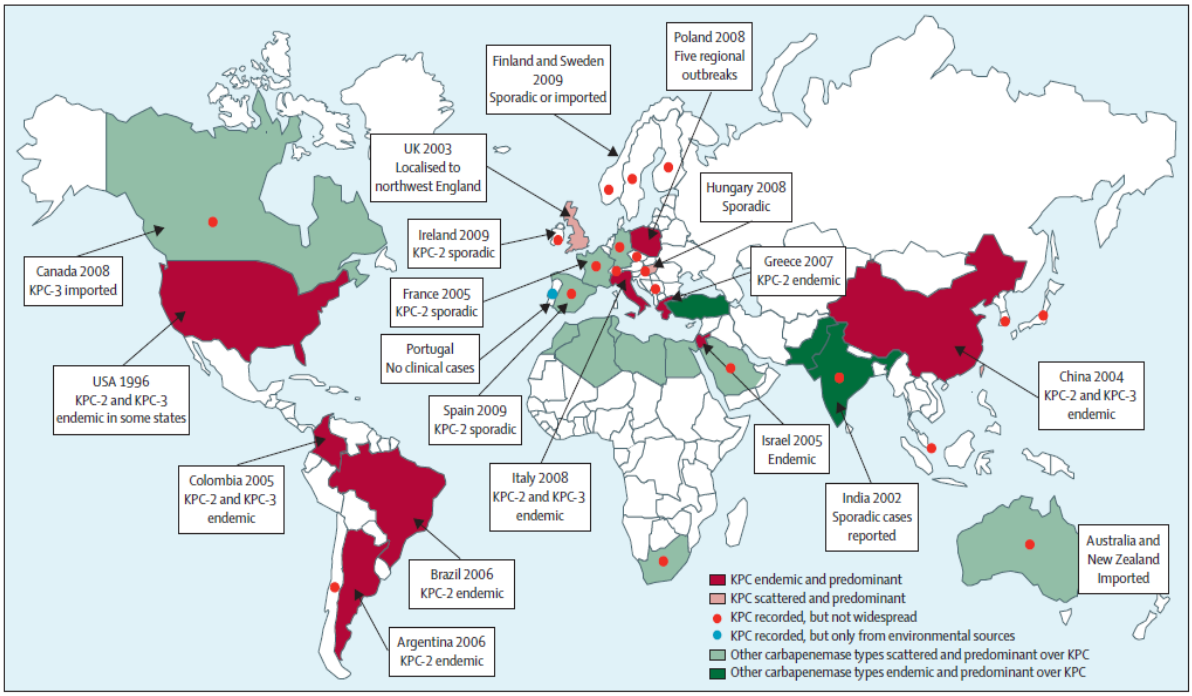

Figure 1.6 - Epidemiological incidence of several types of KPCs producer types by country of origin (Munoz-Price *et al*. 2013).

infections by CRE bacteria, but with variable degree of success (Urban *et al*., 2008). Therefore, these bacteria cause a high mortality rate among patients with bloodstream infections (Munoz-Price, 2013; Clegg & Murphy, 2016).

KPC genes possess a great potential to spread horizontally as some of them are encoded on transposons and often found present on several types of plasmids (Queenan & Bush, 2007). Horizontal transfer is not restricted to *Klebsiella* as KPC genes have been reported on other *Enterobacteriaceae*, including *E. coli, Enterobacter* species, *Salmonella enterica, Proteus mirabilis, Citrobabacter freundii* (Queenan & Bush, 2007; Bush *et al*., 1995; Villegas *et al*., 2005) and *Pseudomonas* species (Munoz-Price *et al*., 2013). *K. pneumoniae* KPC ST258, defined by multilocus sequence typing (MLST) of 7 *loci*, played a major role in disseminating its associated KPC enzymes worldwide (Munoz-Price *et al*., 2013). More detailed information on ST258 and genes concerned in MRDs dissemination can be found in recent reviews (Iredell *et al*., 2016; Paczosa & Mecsas., 2016).

KPC strains incidence has been steadily increasing worldwide (Munoz-Price *et al*., 2013; Iredell *et al*., 2016) (Figure 1.6), as well as ESBLs producing *K. pneumoniae* (Boucher *et al*., 2009), making urgent to find new ways to successfully treat infections caused by these MDRs bacteria.

1.4. The present work objectives

In this dissertation, I will explore the role of BCNs ortholog of *K. pneumoniae* kp52.145 virulent strain, to confer an antibiotic resistance mechanism as described for *B. cenocepacia* (El-Halfawy & Valvano, 2013). In doing so, I aim to demonstrate that BCNs provide general resistance mechanism that can be exploited by important antibiotic-resistant pathogens. Initially I will assess the protection capacity of a recombinant *K. pneumoniae* BCNs protein. Simultaneously, I will attempt to delete BCNs gene from *K. pneumoniae* chromosome. Also, the regulation properties of the same gene under oxidative stress will be evaluated. Finally, I will perform *in silico* studies of *K. pneumoniae* BCNs by comparing with other BCNs from different bacteria. Additionally, synteny studies will be performed.

Here, I will describe all the developed work done until the submission of this dissertation. Additional experimental work is still underway and new data generated will be presented during the public defense.

CHAPTER II – MATERIALS AND METHODS

2.1. General protocols

2.1.1. Bacterial strains and growth conditions

Strains and plasmids used for this thesis are listed on Table S1 (see Supplementary data on CHAPTER V). Bacteria were grown at 37˚C, 180 rpm, in *Difco™ LB* broth. Rhamnose conditional mutants were grown on M9 minimal medium (42 mM Na_2HPO_4, 8 mM NaCl, 10 mM NH_4Cl supplemented with Casamino Acids (80 µg/mL), vitamin B1 (10 µg/mL), tryptophan (40 µg/mL), $CaCl_2$ (20 µM), $MgSO_4$ (200 µM), and 0.5% (w/v) glucose or 0.5% (w/v) rhamnose when

required. Mueller-Hinton media cation adjusted with 10 mg/L Mg^{2+} and 20 mg/L Ca^{2+}, final concentration, was used for MIC determinations. Each medium were added with antibiotics trimethoprim (50 µg/mL for *E. coli* strains; 100 µg/mL for *K. pneumoniae*), ampicillin (100 µg/mL), tetracycline (100 µg/mL for *B. cenocepacia*; 30 µg/mL for *E. coli*; 12.5 µg/mL for *K. pneumoniae*), kanamycin (40 µg/mL) final concentrations when required.

2.1.2. General molecular techniques

K. pneumoniae kp52.145 genomic DNA extraction was carried out using *Genomic DNA Mini kit* (Invitrogen), chromosomal amplicons were generated by PCR using *HotStar Hifidelity polymerase Kit* (Qiagen), using 20% Q-solution final concentration and thermocycled at the following temperatures: 5 min at 95˚C; 35 cycles of 30 s at 95˚C, 30 s at 55˚C and 1 min 72˚C and final extension 10 min at 72˚C and purified by *QIAquick PCR purification kit* (Qiagen). Plasmid extractions were carried out using *QIAprep Spin Miniprep Kit* (Qiagen). DNA restriction endonuclease digestions, plasmid dephosphorylation, ligations reactions and agarose gel electrophoresis were performed according to standard techniques (Sambrook *et al*., 1990). Restriction enzymes and *Antarctic phosphatase* were purchased from *New England BioLabs*, *T4 DNA ligase* from Roche Diagnostics. DNA transformation with *E. coli* strains was carried out by calcium chloride method (Cohen *et al*., 1972). Colony-PCR (cPCR) were carried out with *Taq polymerase kit* (Qiagen) with the following parameters: 3 min at 95˚C; 45 cycles of 15 s at 95˚C, 30 s at 56˚C and 1 min 72˚C; and a final extension at 72˚C for 10 min. PCR products were screened on 0.7% (w/v) agarose gels. Gene sequence of positive transformants was verified by sequencing. All designed primers and respective restriction enzymes are listed on Table S2.

2.1.3. Biparental conjugation

The various constructed vectors from all other procedures, which were transformed in *E. coli* DH-5α and *E. coli* GT115, were extracted and transformed into diaminopimelic acid (DAP) dependent *E. coli* β2163, capable to mobilize the vectors into the recipient strains, such as *K. pneumoniae* or *B. cenocepacia*, by biparental conjugation. The biparental conjugations were carried out using *2,6-Diaminopimelic acid* bought from Sigma-Aldrich®.

Mobilization of the vectors was performed by growing overnight of recipient strain, with 180 rpm orbital shaking and the donor strain without shaking. Next day, both strains were pelleted by centrifugation at 4000 rpm for 20 min, washed in 5 mL of 10 mM $MgSO_4$, pelleted again and resuspended in 500 µL 10 mM $MgSO_4$. A mixture of 100 µL of each strain patched in LB agar supplemented with 0.3 mM of DAP final concentration, incubated overnight at 37˚C. Next day, serial dilutions were made, until 10^{-4}, from the recovered patched biomass and plated on LB without the addition of DAP and with the appropriate antibiotic. The grown colonies were screened by cPCR and/or luminescence on *UVP* (BioSpectrum® AC Imaging System).

2.2. *K. pneumoniae* BCN studies

2.2.1. Cloning, expression and purification of *K. pneumoniae* kp52.145 BCN

K. pneumoniae kp52.145 *yceI* nucleotide sequence (GenBank ID: FO834906.1) and YceI amino acid sequence (GenBank ID: CDO15049.1) was retrieved from *National Center for Biotechnology Information* (NCBI) using *Basic Local Alignment Search Tool* for proteins (BLASTp) algorithm, deploying as query sequence the protein sequence of the putative exported protein from *B. cenocepacia* J2315 (GenBank: CAR53634.1) (Table S3).

To facilitate the differentiation from other BCN orthologues, such as *B. cenocepacia*'s BcnA and BcnB, the gene encoding *K. pneumoniae*'s BCN (*yceI*) will be referred to as *bcnK* and its respective encoded protein will be mentioned as BcnK.

bcnK was amplified by PCR with (primers Q-775 and Q-776) and without (Q-873 and Q-776) its signal peptides, cloned into pDA-CTHis, which contains an N-terminal hexahistidine tag (His-tag), originating pDG1 and pDG5, respectively. To clone into pET28a (+) isopropyl thiol-β-D-galactoside (IPTG) inducible vector, *bcnK* was amplified without its signal peptide and encoding an N- and C-terminal His-tag (Q-873 and Q-880) giving rise to pDG7. Also, *bcnK* was amplified without signal peptide and coding for a N-terminal His-tag (Q-873 and Q-895), and cloned into pET28a (+). Primers (Q-907 and Q-908) for *bcnK* cloning into pUC19 inducible vector were design without *bcnK*'s signal peptide and to contain a His-tag followed by a STOP codon on *bcnK*'s C-terminal giving rise to pDG11. This last vector was point mutated by amplifying (Q-961 and Q-962) to add an adenine base upstream of *bcnK* start codon and digested with *DpnI* overnight at 37˚C afterwards, transformed into *E. coli* and the resulting colonies were selected in ampicillin. The generated amplicons and respective vectors were digested with restriction enzymes, listed on Table S2, ligated and transformed into *E. coli* DH-5α. IPTG inducible vectors were transformed into *E. coli* BL2. Overnight cultures induction were carried out using 0.05 mM IPTG, final concentration and further incubated for 3 h at 25˚C, centrifuged at 10,000 x *g* for 15 min at 4˚C, washed with Tris-buffer 50 mM, pH 7.4 and pelleted again, resuspended in Lysis buffer (Table S4 A) and passed through *One Shot* (E1061, Constant System) at 18 ksi (124.1 GPa). The resulting lysate was centrifuged at 15,000 x *g* for 20 min at 4˚C for cell debris removal and to obtain the total protein fraction. Soluble and membrane protein fractions were obtained by centrifuging the total protein fraction at 30,000 x *g* for 45 min at 4˚C. BcnK purification was carried out by mixing the soluble fraction with coated Ni^{2+} *Chelating Sepharose™ Fast Flow* (GE Healthcare) beads overnight at 4˚C previously treated with equilibration buffer (Table S4 B). Next day, the supernatants were collected, labelled as Flow through, and the beads were washed with increasing concentration of imidazole (50 mM and 75 mM), eluted in 400 mM (Table S4 C) and dialyzed in 4 L of phosphate-buffered saline (PBS) overnight at 4˚C. In each step an aliquot was collected for further analysis. The dialyzed protein was filter sterilized with 0.45 µm *Whatman™* (SPARTAN Syringe Filter) and conserved at -80ºC until used. The presence of the protein was confirmed by SDS-PAGE and Western-Blot.

2.2.2. SGS-PAGE & Western-blot

The various protein fractions obtained were boiled at 100˚C for 10 min, loaded in a 16% SDS-PAGE gel, run for 75 V for 35 min and 130 V for 2 h. For SDS-PAGE staining the gel were dyed with *PAGE-Blue*™ (Thermo Scientific) for 2 h and distained overnight.

For Western-blot assays, the protein transfer was carried out using *Biorad Trans-Blot® Turbo™ Kit* into a nitrocellulose membrane for 20 min with 1.3 A and 25 V, blocked overnight at 4˚C with *Blocker™ Casein in TBS* (Thermo Scientific). Next day, the membrane was washed with Tris-buffered saline (TBS), the primary antibody *Anti-His Antibody* (GE Healthcare Life Sciences) was added diluted 1:3000 and incubated at 4˚C for 2 h, washed three times with TBS, added the secondary antibody *AlexaFluor® 680 anti-mouse IgG* (Life Technologies) diluted 1:20000, incubated for 45 min, washed three times and checked at *Li-cor* (Odyssey®) at the wavelength of 700 nm.

2.2.3. *P. aeruginosa* polymyxin B (PmB) protection assays

The purified and dialyzed BcnK obtained from the expression of the various constructed vectors, were concentrated if required using *Vivaspin 500* (3000 MWCO PES, Sartorius Stedim Biotech), quantified by *NanoVue Plus™ Spectrophotometer*. Overnight cultures of *P. aeruginosa* PAO1 were subcultured for 2 hours, OD_{600} adjusted to 0.04, loaded in a 100 well honeycomb plate along with 2; 1; 0.5; 0 µg/mL final concentration of PmB and 1.5 µM, final concentration, of purified BcnK. Controls were performed with the same antibiotic concentrations using PBS instead of purified BcnK. Each antibiotic concentration, with and without BcnK, was tested within triplicates. The OD_{600} was read each hour at 37ºC for 24 h on *Bioscreen C* (Oy Growth Curves Ab Ltd.).

2.3. *K. pneumoniae* mutagenesis

2.3.1. *bcnK* deletion in *K. pneumoniae* kp52.145

Unmarked deletion method was performed as previously described (Flannagan *et al*., 2008). To delete *bcnK*, PCR amplifications of ≈ 300 bp flanking regions *bcnK* were performed (Q-786 and Q-787; Q-788 and Q-789). Amplicons were digested with *XbaI-XhoI* and *XhoI-EcoRI* respectively and cloned into pGPI-*SceI*-2 digested with *XbaI-EcoRI* giving rise to pDG4. Simultaneously, it was also created a vector containing ≈ 1000 bp flanking regions *bcnK* (Q-810 and Q-811; Q-812 and Q-813), the amplicons were joined together using *Ex Taq® DNA polymerase* (TaKaRa) and ligated into *pGEM®-TEasy* resulting on the vector *ΔyceI*-pGEMT. The vector was digest with *EcoRI*, gel purified and ligated with pGPI-*SceI*-2 giving rise to pDG2. Each plasmid was introduced to wild type (WT) strain of *K. pneumoniae* kp52.145 by conjugation, separately, and selected with trimethoprim. The resulting conjugants were subjected to a new conjugation with pGPI-*SceI*-SacB and selected through tetracycline resistance.

Insertional inactivation was performed by cloning an *bcnK* internal fragment with 297 bp (Q-902 and Q-903) into suicide vector pGPΩTp (Flannagan *et al*., 2007) which contains *dhfr*

flanked with Ω-fragments, which when conjugated into *K. pneumoniae*, creates a polar mutation stopping *bcnK* transcription. The constructed vector was named pDG9.

The procedures described above were carried out in both aerobic and anaerobic conditions. For anaerobic conditions, samples were manipulated and incubated in *Whitley A35 Anaerobic Workstation* under an atmosphere composed by 10% CO_2, 10% H_2 and 80% N_2.

2.3.2. Rhamnose conditional promoter and essentiality assessment

The vector pSCrhaB2-e-GFP (Cardona *et al*., 2006) was conjugated into *K. pneumoniae* kp52.145 WT. The exconjugants were selected in LB supplemented with trimethoprim. Ten colonies were picked and each grown overnight at 37ºC in M9 medium supplemented with 0.5%, 0.2% of rhamnose or 0.5% of glucose. Next day, the bacterial suspensions were deposited in slides covered with thin layer of 0.8% (w/v) agarose and observed by light and fluorescent microscopy. The images were acquired using *Axioscope 10* (Carl Zeiss) microscope coupled to a camera *AxioCam MRm* (Zeiss) and an endow GFP bandpass emission filter set with the 470 ± 25 nm emission range, 525 ± 25 nm excitation. Images were digitally processed using *ZEN 2012* (Blue Edition) Service Pack 1 image software.

The rhamnose conditional promoter construct was performed as previously described (Ortega *et al*., 2007) by cloning a 306 bp fragment (Q-775 and Q-904) spanning from the 5' region of *bcnK* into pSC200, giving rise to pDG10. The resulting vector possesses a rhamnose-inducible P_{rhaB} upstream of the multiple cloning site, enabling to drive the expression of *bcnK* in the presence of rhamnose and repressing it in glucose. The vector's conjugation was performed in LB supplemented with 0.5% rhamnose and selected in LB with 0.5% rhamnose and trimethoprim. The conditional mutants were grown overnight at 37ºC in M9 medium with 0.5% rhamnose, spun down and washed three times with PBS, resuspended in PBS and adjusted to an OD_{600} of 1 (Neat solution). Drops (10 µL) of Neat, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} dilutions were plated in M9 agar square plates supplement with 0.5% (w/v) rhamnose or 0.5% (w/v) glucose and incubated at 37ºC. The strains XOA10 and XOA12 were used as a negative and positive control, respectively.

2.4. Oxidative stress studies

2.4.1. Transcriptional fusions to *luxCDABE* and luminescence assays

Promoter region of *bcnK*, was amplified by PCR (Q-909 and Q-910). The amplicon with ≈900 bp was digested and cloned into the digested and dephosphorylated pGSVTp-*lux* suicide vector and transformed into *E. coli* GT115. The primers for *oxyR* and *waaE* (Q-841 and Q-842; Q-852 and Q-853, respectively) were designed after ELIC method (Koskela & Frey, 2015) containing ≈25 bp homologous with the vector's region adjacent to region where it will be cloned in pGSVTp-*lux*. The amplicons and digested vector were quantified in *NanoVue Plus™ Spectrophotometer*, mixed in a ratio of 3:1, respectively, in a final volume of 10 µL of Mili-Q water and incubated for 1 h at room temperature and transformed into *E. coli* GT115. The transformants colonies were selected in LB with trimethoprim, grown overnight and checked for luminescence on *POLARstar® Omega* for 12 h at 37ºC treated with and without paraquat 1.5 µM final

concentration. From the colonies that displayed luminescence, three of each construct were chosen and the vector was extract and transformed into *E. coli* β2163 and conjugated into *K. pneumoniae* and selected in LB with trimethoprim. The obtained exconjugants were checked for luminescence, grown overnight and luminescence and OD_{600} were measured in the presence/absence of serial dilution of paraquat (10; 5; 2.5 and 0 µM) for 12 h at 37ºC on *POLARstar® Omega*. The expression levels of each gene of interest in the different strains background were calculated as Relative Luminescence Units per OD_{600} (RLU/OD_{600}) for each condition.

2.5. *B. cenocepacia* BCN complementation and MIC assays

B. cenocepacia ΔBcnAΔBcnB were complemented with pDG1 and pDA-CTHis through biparental conjugation and selected in LB with tetracycline giving rise to DNG4 and DNG5, respectively. *B. cenocepacia ΔBcnAΔBcnB*; DNG4 and DNG5 MIC's were tested accordingly with (Wiegand *et al*., 2008). The strains were grown overnight in Mueller-Hinton cation adjusted, OD_{600} adjusted to 0.005, inoculated in 100 well honeycomb plate with serial dilutions from 2048 µg/mL to 8 µg/mL of rifampicin. The plates were incubated for 24 h at 37ºC on *Bioscreen C*. Triplicates were performed for this assay.

2.6. BcnK genomic studies

The previously retrieved BcnK amino acid sequence from NCBI was used to perform the following BCN genomic studies. *K. pneumoniae* kp52.145 BcnK amino acid sequence was compared against *Klebsiella* genus (taxid: 570) using BLASTp and selecting non-redundant protein sequences search. Amino acids sequence alignment and phylogenetic data were generated by *Clustal Omega* v1.2.2 (Sievers *et al*., 2011). Neighbour-joining cladograms were generated from the alignment data using *FigTree* v1.4.2 software. The domain structure of BcnK was accessed on *Conserved Domain Database* (CDD) tool on NCBI. Neighbourhood gene studies were performed by *SyntTax* (Oberto, 2013). Interaction of BCNs (YceI's) protein with other proteins and molecules was accessed using the *Search Tool for the Retrieval of Interacting Genes/Proteins* (STRING) (Szklarczyk *et al*., 2014). Amino acid sequence from *B. cenocepacia* CybB (GenkBank ID: CAR53635.1) was also retrieved from NCBI.

2.7. Computational methods

K. pneumoniae kp52.145 BcnK tridimensional (3D) folding prediction was generated in *Protein Homology/analogy Recognition Engine* V 2.0 (Phyre2) (Kelley *et al*., 2015) using amino acidic sequence and visualized in Jmol v13.0. BcnK molecular weight was predicted on *ExPASy*, Bioinformatics Resource Portal translation tool (Gasteiger *et al*., 2005). BcnK signal peptide prediction was performed on *SignalP* 4.1 Server in CBS website (Petersen *et al*., 2011). DNA sequence analysis and vectors figures were generated in *SnapGene*™ 1.1.3. Statistical analysis was performed with *GraphPad Prim 6*.

CHAPTER III – RESULTS AND DISCUSSION

3.1. Activity of purified BcnK in an antibiotic protection assay

BcnK was expressed in several vectors to obtain a pure functional protein for downstream assays. I first purified BcnK expressed in the pDA-CTHis vector (pDG1; Table S1) using Ni^{2+}-affinity chromatography followed by SDS-PAGE and Western-blot, which revealed a polypeptide band with a predicted molecular weight of 19.6 kDa (Figure 3.1). This protein was employed in an antibiotic protection assay using *P. aeruginosa* as a reporter strain. In the presence of BcnK, *P. aeruginosa* PAO1 showed increased resistance to PmB (approximately 65.5% increase in the $\%OD_{600}$) (Figure 3.2A). Although protection was an expected results, this assay was performed

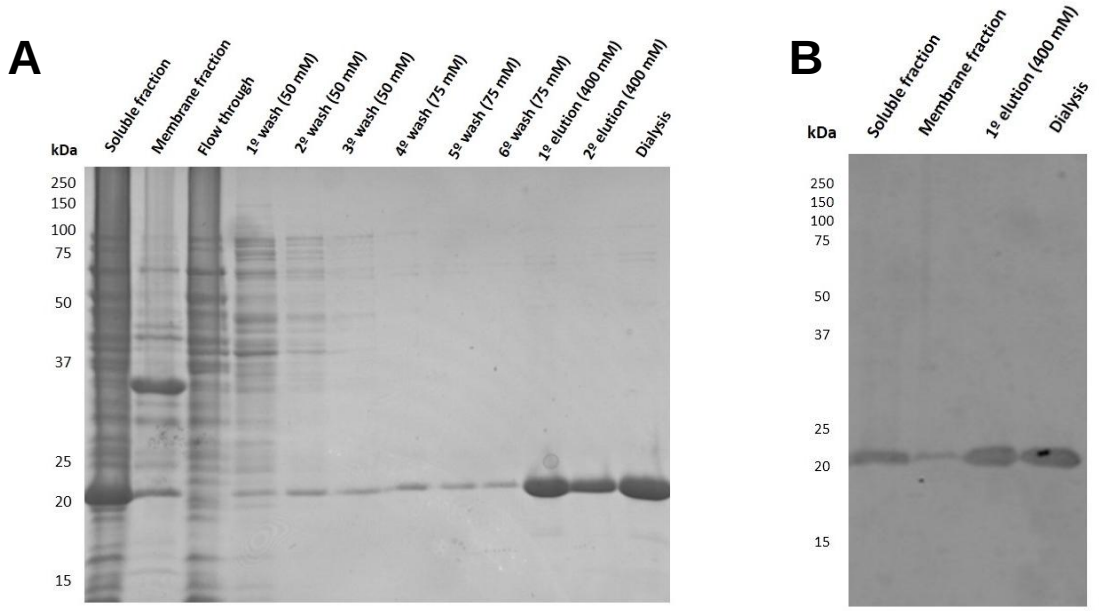

Figure 3.1 – Verification of BcnK presence expressed from pDG1 during several steps of -Ni^{2+} chromatography purification procedure (**A**) SDS-PAGE of the soluble and membrane protein fractions; purification column flow through; washes with imidazole and elution of BcnK bound to the column, performed with the respective and concentrations of imidazole and the respective dialysis in PBS; (**B**) Western-Blot performed on crucial steps of BcnK purification.

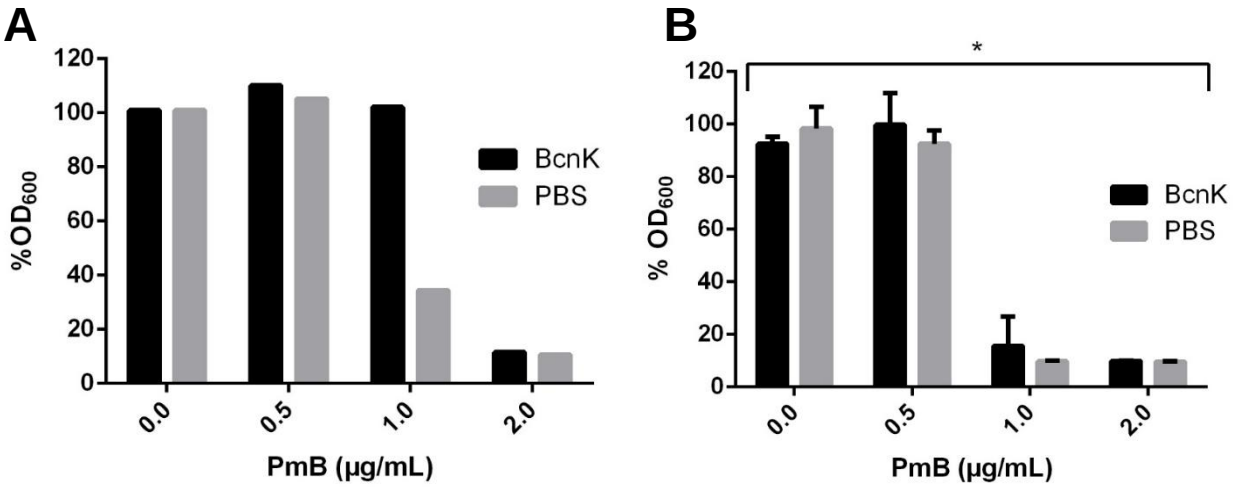

Figure 3.2 – *In vitro* protection assay of *P. aeruginosa* against PmB with 1.5 µM of BcnK. (**A**) BcnK expressed from pDG1 (n=3; from an individual assay); (**B**) BcnK expressed from pDG8 (n=9; from 3 independent assays). Results correspond to the end points of 24 hours incubation shown in $\%\ OD_{600}$ relative to untreated control. Significant differences (* $P<0.0001$) were tested using 2way ANOVA**.**

only once, as this protein rapidly formed aggregates during the purification and dialysis in PBS that precluded repeating the assay for statistical analysis.

The rapid aggregation of purified BcnK made difficult to verify the actual amount of protein in the protection assay. BcnK aggregation may result from the lipid moiety N-acyl-S-sn-1,2-diacylcerylcysteine present in BcnK N-terminus (Bishop, 2000), which is hydrophobic and enables BCNs to anchor in the bacterial membrane. Thus, dialyzing the purified protein in a polar solvent, such as PBS, likely resulted in aggregation. The same behavior was observed when BCNs orthologues from *B. cenocepacia* were purified from constructs containing the respective signal peptides (El-Halfawy & Valvano, 2013). To solve such obstacle, *B. cenocepacia* BCN orthologues were purified from constructs devoid of their signal peptides. The same approach was adopted here; *bcnK* gene was amplified without its predicted signal peptide (Figure S1) and cloned into the same backbone as pDG1, giving rise to pDG5. However, this construct resulted in reduced protein quantities after purification. To increase the protein expression in *E. coli*, two additional constructs containing *bcnK* without its signal peptide were cloned, giving rise to pDG7 (containing N- and C- terminus His-Tag) and pDG8 (containing a N-terminus His-Tag), were created using the IPTG inducible pET28a (+) vector. These constructs afforded sufficient quantities of protein expression for the purification based on SDS-PAGE and Western-blot of crude bacterial lysates (data not shown). However, protein obtained from pDG7 expression still aggregated, while that from pDG8 remained stable upon dialysis in PBS. The different solubility observed between these two recombinant proteins could depend on the His-tag's (Woestenenk *et al*., 2004). While pDG7 encodes BcnK with two His-tag's, (one on N- and the other C-terminal), pDG8 encodes a protein with only one His-tag at the N-terminus. Yet, no detectable protection in *P. aeruginosa* protection assay was found with the pDG8 construct (Figure 3.2B). From the comparison of the results with the full-length mature and lapidated protein expressed from pDG1, we hypothesized that the His-tag position in the recombinant protein could have an effect on its activity. In pDG1, BcnK has a C-terminus His-tag, while in pDG8 BcnK is expressed with an N-terminal his-tag, which may interfere with antibiotic binding. A new construct expressing a protein with a C-terminal His-tag was then created (pDG11). However, no BcnK protein could be found by SDS-PAGE of the crude lysates (data not shown). Analysis of the pDG11 DNA sequence indicated that a putative ribosomal binding site (Shine-Dalgarno, SD) sequence was too closely positioned to the start codon of *bcnK* (3 nucleotides upstream). The SD is responsible for the ribosome binding to the transcribed mRNA and typically should be positioned at 7-11 nucleotides of the translation start site (Chen *et al*., 1994). Because in pDG11 there is an upstream encoding region, *lacZα*, which possesses a SD sequence within 7 nucleotides from its start codon, we attempted to correct the reading frame by site-directed mutagenesis to create a LacZα-BcnK fusion that would be correctly expressed (Figure S2). Unfortunately, several colonies containing the putative translation-corrected *bcnK* chimeric gene failed to express the protein, verified by SDS-PAGE analysis.

We then hypothesized that the absence BcnK of activity in the protection assay could be related to BcnK misfolding. The expression of BcnK without signal peptide targets the BcnK protein into the cytosol, instead of the periplasmic space, where BcnK is predicted to locate

originally. The presence of the recombinant protein in the cytosol may result in protein misfolding (Dyson *et al*., 2004; Luan *et al*., 2004; Braun *et al*., 2002) This would explain the both lack of expression, as misfolded proteins are generally subjected to degradation by proteolysis (Kopito, 2000; Baker & Sauer, 2006; Tomoyasu *et al*., 2001) and production of protein in a misfolded conformation may prevents antibiotic binding. Other possible explanation for the lack of expression is the formation of insoluble inclusion bodies (IBs), which is often observed when foreign genes are overexpressed in *E. coli* (Tsumoto *et al*., 2003; Lemercier *et al*., 2003). These IBs can be separated from the cell lysate by low speed centrifugation (2000 x *g*) (Hancock, 2001). However, we could not detect IBs in our lysates. Therefore, in future subsequent experiments, we propose to overcome the lack of expression by using pRF130 (Flannagan *et al*., 2007). This vector provides a cleavable signal peptide, which would help direct unlipidated, mature BcnK to the periplasmic space, where the protein should fold normally.

In conclusion, although we initially observe BcnK protection of *P. aeruginosa* against PmB, this result could not be repeated with other constructs likely due to problems with protein expression and misfolding.

3.2. The *K. pneumoniae bcnK* gene seems to be essential for bacterial viability

To investigate the role of BcnK in *K. pneumoniae* antibiotic phenotype resistance we attempted to construct an unmarked bcnK gene deletion. For this purpose, I constructed the mutagenesis plasmid pDG4, which was employed for deletion mutagenesis as described in Material and Methods. However, no mutants were obtained after repeated attempts. In all cases, screening of the colonies though cPCR indicated only merodiploids (Figure 3.3).

Therefore, we attempted to obtain a polar mutation in *bcnK* using pDG9. The rationale of this approach was to amplify an internal fragment of the *bcnK* coding region, which was cloned into the suicide pGPΩTp vector (Flannagan *et al*., 2007). The integration by homologous recombination of the resulting plasmid (pDG9) in the *bcnK* gene would result in a strong polar mutation due to the presence of the Ω-fragments in the vector that interrupt transcription in both directions (Flannagan *et al*., 2007). Again no mutants were obtained through this method.

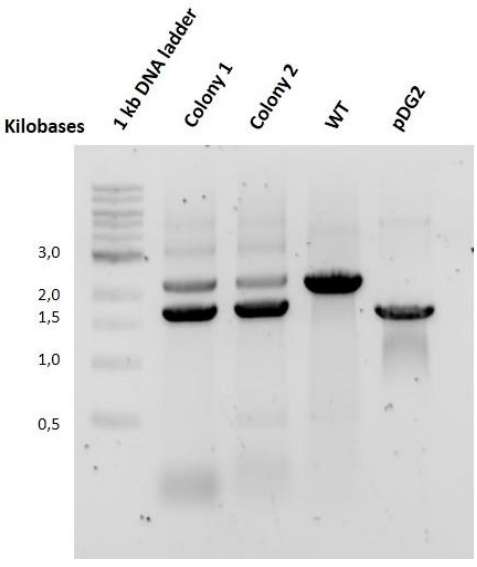

Figure 3.3 – cPCR performed of *K. pneumoniae* trimethoprim sensitive colonies obtained after the second conjugation of *bcnK* of unmarked deletion using pDG2. *K. pneumoniae* merodiploid colonies 1 and 2 were compared against *K. pneumoniae* WT purified chromosome and the purified knockout plasmid pDG2.

Failure to obtain gene deletion or a polar mutation of *bcnK* suggested the possibility that this gene is essential for the viability of *K. pneumoniae*. Other BCN genes have been proposed to be essential in *Salmonella enterica* serovar Typhimurium (Knuth *et al*., 2004) and in *Vibrio cholerae* (Cameron

et al., 2008). In contrast BCNs orthologues in *B. cenocepacia* (El-Halfawy & Valvano, 2013), *Neisseria meningitis* (Donnarumma *et al*., 2015) and *E. coli* (Gerdes *et al*., 2003) are not essential.

To verify the essentiality of *bcnK* in *K. pneumoniae*, we employed a strategy based on placing a rhamnose inducible promoter upstream of the chromosomal copy of the *bcnK* gene. First, we had to demonstrate that this conditional promoter works in *K. pneumoniae*. To test it, pSCrhaB2-e-GFP was conjugated into *K. pneumoniae*. This vector encodes a rhamnose-inducible gene encoding the green fluorescent protein (GFP). GFP expression by this vector is suppressed in the presence of glucose. Figure 3.4 shows the different levels of fluorescence in *K. pneumoniae* expressing eGFP encoded from the vector pSCrhaB2-e-GFP. No visual differences are observed between bacteria growing in 0.2% and 0.5% rhamnose, showing that the rhamnose promoter is highly active irrespective of these rhamnose concentrations. However, fluorescent bacterial cells were not observed in cultures grown with glucose, indicating that the rhamnose promoter is inactive as expected and also observed in *B. cenocepacia* (Cardona *et al*., 2006). Based on this information, we constructed pDG10, a derivative of pSCrhaB2-e-GFP where the GFP gene was replaced by *bcnK*. This plasmid was mobilized into the *K. pneumoniae* strain and the resulting recombinants, putatively containing a *bcnK* gene under the control of the rhamnose inducible promoter, were screened in M9 medium supplemented with glucose or rhamnose. However, all the colonies investigated could grow in both media (Figure 3.5), suggesting that *bcnK* is either not essential for *K. pneumoniae* viability or a very low level of constitutive expression in the absence of rhamnose is sufficient to overcome loss of viability.

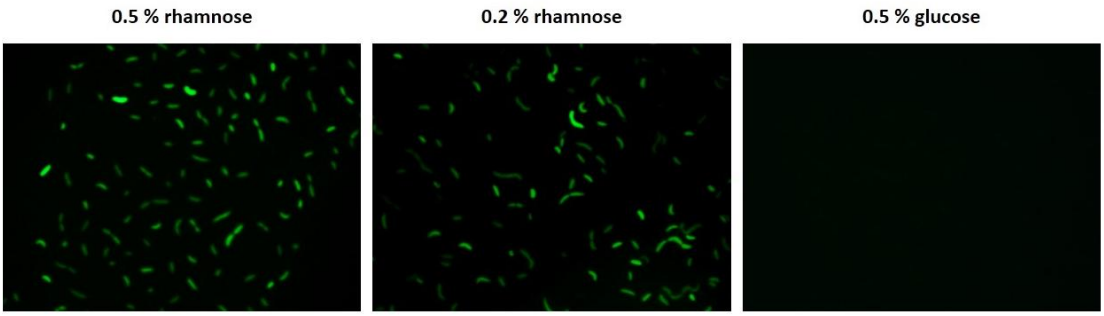

Figure 3.4 – Microscopy of *K. pneumoniae* exconjugants expressing eGFP under the rhamnose inducible promoter. Bacteria were grown at the indicated concentrations of rhamnose and glucose.

Future experiments will involve attempting to construct an unmarked deletion of *bcnK* in the presence of a complementing plasmid, with an exogenous BCN (that differs in nucleotide sequence with *bcnK*), and submitting the complemented bacteria to the unmarked deletion mutagenesis method. This method aims to show that, from complemented *K. pneumoniae*, *bcnK* can be deleted. If so, the further removal of the complementation plasmid from the defective mutants should demonstrate if *bcnK* is indeed essential for *K. pneumoniae* viability.

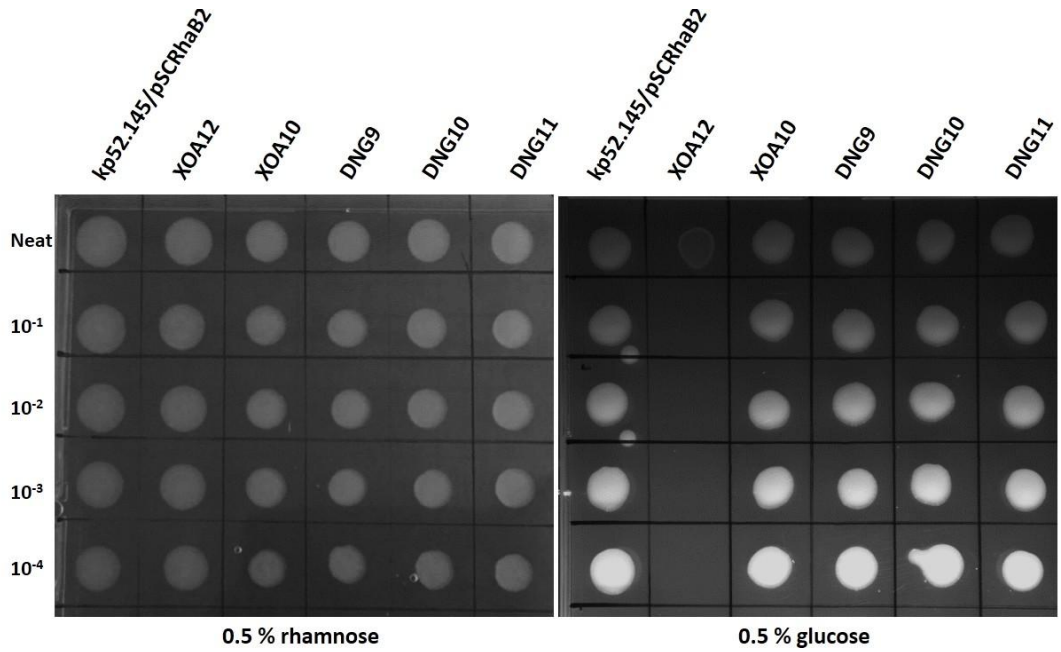

Figure 3.5 – *K. pneumoniae bcnK* essentiality assessment using the obtained exconjugants containing rhamnose inducible promoters (DNG9-11). *K. pneumoniae* wild type was complemented with pSCrhaB2 (kp52.145/pSCrhaB2) using rhamnose inducible promoter as control. XOA12 and XOA10 were used as rhamnose-dependent and rhamnose-independent control mutant (Ortega *et al*., 2007). Bacteria were grown on the indicated rhamnose and glucose concentrations.

3.3. *bcnK* regulation under various stress conditions

This study also involved determining the regulation of *bcnK* under oxidative stress. Previously, El-Halfawy & Valvano (unpublished) demonstrated, using luciferase reporter plasmids, that *B. cenocepacia* BcnA expression is upregulated by oxidative stress. Many antibiotics induce oxidative stress when bacteria are exposed to sublethal concentrations (Albesa *et al*., 2004). If BcnK protein plays a role in antibiotic resistance, one could predict that it is also upregulated under the same conditions. We then applied the same luciferase method, as the one used for *B. cenocepacia*, by cloning the promoter regions of *bcnK*, upstream of a *luxCDABE* cluster present in the suicide vector pGSVTp-lux.

To evaluate *bcnK* expression levels under oxidative stress, we constructed P_{bcnK}*::luxCDABE* and P_{oxyR}*::luxCDABE.* The latter construct was used as a positive control for oxidative stress since *oxyR* responds to oxidative stress (Honma *et al*., 2009; Loprasert *et al*., 2002; Seib *et al*., 2007). As a negative control, we constructed P_{waaE}*::luxCDABE.* The *waaE* gene encodes a protein needed for the inner-core lipopolysaccharide in *K. pneumoniae* 889 (Izquierdo *et al*., 2002), and is not stimulated by oxidative stress. Expression was examined by determining luminescence arising from the expression of the lux operon (Winson *et al*., 1998).

These assays are still under way, but preliminary results obtained from stimulation with paraquat (a compound that elicits superoxide stress) in bacteria containing P_{bcnK}*::luxCDABE* and P_{oxyR}*::luxCDABE* suggest that *bcnK* is upregulated (data not shown), as it was previously reported for *bcnA* (El-Halfawy & Valvano, unpublised). Further studies will include antibiotics such as rifampicin, norfloxacin and ceftazidime which are known to induce oxidative stress in the bacteria.

3.4. *B. cenocepacia* BCN complementation

A *B. cenocepacia ΔbcnA* mutant shows increased susceptibility to PmB, rifampicin and norfloxacin (El-Halfawy & Valvano, 2013). Previous studies showed that BCNs from *P. aeruginosa*, *Mycobacterium tuberculosis and S. aureus*, could complement this mutant, indicating that these proteins have the same function as BcnA in conferring antibiotic resistance (El-Halfawy & Valvano, unpublished). In our study, the vectors pDG1, which encodes a full-length BcnK protein, was conjugated into *B. cenocepacia ΔBcnAΔBcnB*, resulting in strains DNG4 and DNG5, respectively. These complemented mutants were assessed for MIC against rifampicin, as it was done for BCNs from other species. The results (Table 3.1) showed increased MIC for complemented strains relative to non-complemented. However, this increased MIC values did not differ between pDG1 and the plasmid vector control. El-Halfawy & Valvano (unpublished) used a different vector, pSCRhaB2, to complement the *B. cenocepacia* mutant. Such vector will be used in future complementation assays to clone *bcnK*, under the same conditions as in *B. cenocepacia* study.

Table 3.1 – Rifampicin MIC determination of non-complemented *B. cenocepacia* (WT); non-complemented *B. cenocepacia ΔbcnAΔbcnB* (OME4); *B. cenocepacia ΔbcnAΔbcnB* complementation with *bcnK* (pDG1) and *B. cenocepacia ΔbcnAΔbcnB* complemented with pDG1 backbone (pDA-CTHis). Results expressed in growth **(+)** and no growth **(-)** (n=9, 3 independent experiments).

Rifampicin MIC				
Concentration (µg/mL)	Strains			
	WT	OME4	pDG1	pDA-CTHis
512	-	-	-	-
256	-	-	-	-
128	+	-	+	+
64	+	+	+	+
32	+	+	+	+
16	+	+	+	+
8	+	+	+	+
0	+	+	+	+

3.5. BCN *in silico* structural and genomic characterization of BCNs

3.5.1. BcnA vs BcnK secondary structure comparison

BCNs are a family of proteins with a β-barrel shape followed by an α-helix. BcnK 3D structure was modeled with the Phyre2 webtool. Figure 3.6 shows a prediction of the conserved secondary structure of the BCNs protein family, modelled with 100.0% confidence and 77.0% of amino acid sequence coverage.

Potential BCN active amino acids were searched with the CDD webtool. However, no active residues were registered on this database. El-Halfawy & Valvano (unpublished) generated a BcnA/norfloxacin docking model. They showed that aspartic acid 82 (Asp) and Asp93, when replaced by alanine (a small nonpolar amino acid), resulted in reduced BcnA antibiotic binding activity. Using the same model, BcnA and BcnK were aligned by Clustal Omega (Figure 3.7) in an attempt to identify putative amino acids responsible for antibiotic binding. The amino acid alignment revealed that valine 107 (Val107) and glutamic acid 118 (Glu118) in BcnK correspond to BcnA's Asp82 and Asp93, respectively. 3D predictions in Phyre2 showed that both amino acids assume the same position on the protein structure. Despite the differences, Asp and Glu residues are both polar and negatively charged amino acids. However, Val is a hydrophobic residue. This suggests that other residues on the rim, or slightly inside of the β-barrel, may also play a role in

antibiotic binding. However, the 3D predictions may not be completely accurate, as the homology detection algorithm to build 3D models with the Phyre2 webtool (Kelley *et al.*, 2015), may generate false positives even with proteins that are confidently aligned (Marks *et al*., 2012).

Due to time constrains the role of identified amino acids could not be tested in this study. Nonetheless, these observations may guide future studies to identify key residues in BcnK by performing alanine site-replacement mutagenesis on the identified residues. Alanine consists on a small mass non-charged amino acid which rarely compromises the protein tertiary structure. Also, to identify other important residues, BcnK/antibiotic docking models would be performed. The resulting mutated protein would be used for antibiotic protection and fluorometric Nile Red binding assays.

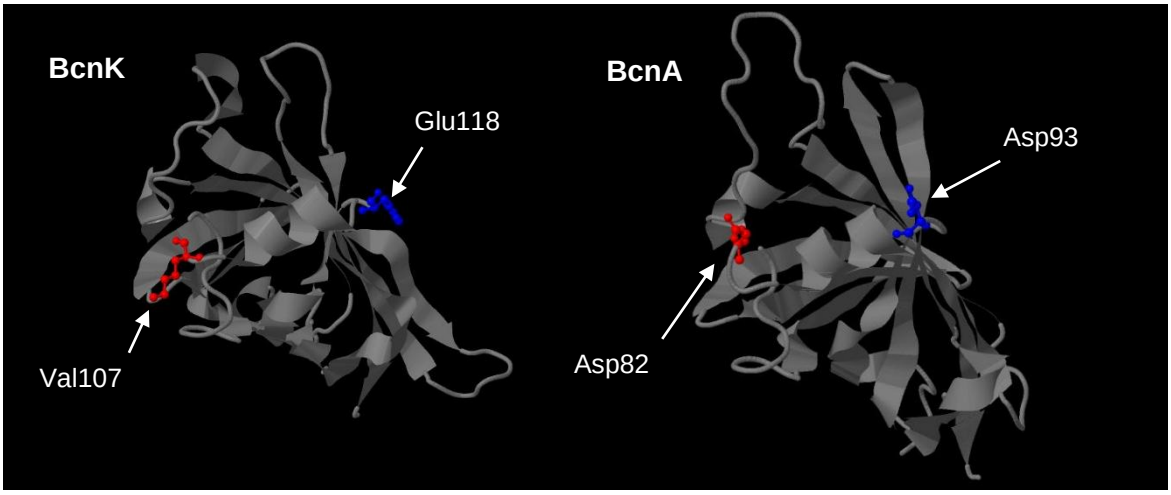

Figure 3.6 – 3D prediction of BcnK (left) and BcnA (right) visualized in Jmol. In red, are shown the corresponding residues for BcnK's Val107 and BcnA's Asp82 and in blue the corresponding BcnK's Glu118 and BcnA's Asp93.

```
BcnK   MFSISPVFFWANIYVPADFEDYCVNTLKKTALLSVLALYIPVSQAAAKEYSLDPQHTSVV
BcnA   --------------MKVSFSRSMLTAFAAAAL--------VASGAAHADVDLAKSKVSAV
                     : ..*.    :.::   :**          .* **   : .*   .:.*.*

BcnK   ISWNHFGFSNPTAYISDVSGKLAFDKENPEKSSVNVTLPVKTIDAHVKALTDEFLGKEYF
BcnA   --SKQMNVP-TEGAFKKFSAQVKFDPAKAAQGSAQMTIDVASFDLGDKMYNDQVAGKDWF
          ::: .     . :...*.:: **   :  :.*.::*: * ::*    *   .*:. **: :*

BcnK   DVKTFPNATFQSTKVESKGDNKYDVEGNLTIKGITKPVVLHAVLNKQDMHPMVKKEAIGF
BcnA   DAKTYPQATFVSSAIAPAGGNKYNVTGKLTIKGKAETVTVPVTVAQNGA-------TQTF
       *.**:*:*** *: :    * ***:* *:***** :: *.: ..: ::          :  *

BcnK   DATGVIKRSDFKLDKY----VSAVSDNVTITLSTEAYAK
BcnA   DGVLPIKRSAFNVGTGEWKDTSIVADEVQIKFHLVATK-
       *..  **** *:: .      .* *:*:* *.:   *
```

Figure 3.7 – BcnK and BcnA amino acid sequence alignment obtained from Clustal Omega. In blue is BcnK's residue, Val107, corresponding to BcnA's Asp82 and in red is BcnK's residue, Glu118, corresponding to BcnA's Asp93. The following symbols stand for **(*)** identical residues; **(:)** identical charge; **(.)** identical property (hydrophobic or hydrophilic).

3.5.2. *Klebsiella* spp. BcnK genomic comparison

To compare BcnKs from *Klebsiella* species, BLASTp analyses were performed using all available *Klebsiella* (taxid: 570) genomic sequences. Clustal Omega was used to align the BLASTp hits (Table S5) and a cladogram was generated by FigTree (Figure S3).

BLASTp revealed proteins with amino acid sequence homologies that varied between 99% and 81% among 100 homologous identified sequences in *Klebsiella* species. The maximum difference of identities between homologous consisted of 24 residues. The cladogram shows that *Klebsiella* BCNs can be divided into 3 clusters. Due the lack of knowledge of the active residues of this protein family, we could not clarify if the observed difference in the residues among *Klebsiella* homologs plays a role in antibiotic binding activity. However, these alignments provide a tool for further studies of protein structure, prediction of antibiotic binding activity, functionality and evolutionary studies.

3.5.3. *bcnK* neighborhood studies

A neighboring gene comparison was done to evaluate and compare the BCNs conservation in *K. pneumoniae* genome and other species using SyntTax with BcnK as query.

Figure S4 shows that the *bcnK* position is highly conserved on several *K. pneumoniae* strains. Also, in *K. pneumoniae* genomes, *bcnK* is isolated from other genes, ≈ 700 bp both upstream and downstream with no coding regions. The analysis for the ESKAPE isolates showed that BCNs genes are highly conserved in each bacterial species (Data not shown). No synteny was found for *B. cenocepacia* and *E. feacium*. However, *yceJ* or *cybB*, which encodes for a putative cytochrome b561 homologue was found associated to BCN genes from several species, such as *P. aeruginosa*, *B. cenocepacia*, *E. coli* and some *Enterobacter* species. In contrast, these genes were not associated to BCNs in *K. pneumoniae*, *A. baumannii* and *S. aureus* (Table 3.2). It is also important to notice that no synteny and BCNs homologous (searched by BLASTp) was found in *E. faecium*. The implications of these findings is discussed below (see section 3.8).

3.8. A general hypothesis for BCNs cellular function

BCN antibiotic binding activity has been studied in the Valvano's lab. Recent unpublished data (Naguib & Valvano) show that BcnA's antibiotic binding capacity is outcompeted by vitamin E, which binds more strongly to BcnA then antibiotics, thus displacing the antibiotics from the protein and increasing their freedom to exert inhibition of bacterial growth.

Structural studies in *Thermus thermophilus* (Handa *et al*., 2005) suggests that BCNs may play an important role in isoprenoid quinone (IQ) metabolism and/or transport and/or storage, as these proteins were isolated with polyisoprenoid side chain buried inside BCNs β-barrel. These side chains, which vary in length depending on the species (Collins & Jones, 1981), are precursors of IQ (Søballe & Poole, 1999). IQ consists on essential components electron-transport chain (ETC). IQ is found in membranes of both prokaryotes and eukaryotes, acting as electron and proton shuttles between complexes I or II to complex III on ETC pathway (Nowicka & Kruk, 2010) (Figure 3.8). It is important to mention that enzymes other than complex I, II or III can

oxidize or reduce IQ, therefore ETC can be branched (Rasmusson *et al*., 2008). Overall, ETC is responsible for adenosine triphosphate (ATP) production under both aerobic and anaerobic conditions, depending on the final electron acceptor, creating a flow of electrons, thus generating proton motive force (Figure 3.8). Also, ETC represents an important mechanism for oxidative stress and gene regulation. Here, it will be focused the role of ETC on oxidative stress control.

Reactive oxygen species (ROS) are toxic byproducts of aerobic metabolism and also they are formed due to environmental stress (Cabiscol *et al*., 2010). ROS are subsequently scavenged by several mechanisms of which IQ, known for its antioxidant activity (Do *et al*., 1996), is an example. Søballe *et al*., (1999) Lagendijk *et al*., (1996) suggest that IQ antioxidant properties are dependent on its high concentrations and its reduced ratio, protecting against lipid peroxidation, DNA oxidation from free ROS and modification of membrane proteins (Ernster & Dallaner, 1995). Condensing this information with the function suggested by Handa *et al*., (2005) and BCN upregulation under oxidative stress (El-Halfawy & Valvano, unpublished), it is now suggested that BCN may function by providing polyisoprenoid chains to the IQ pool, thus decreasing the cell sensibility to oxidative stress.

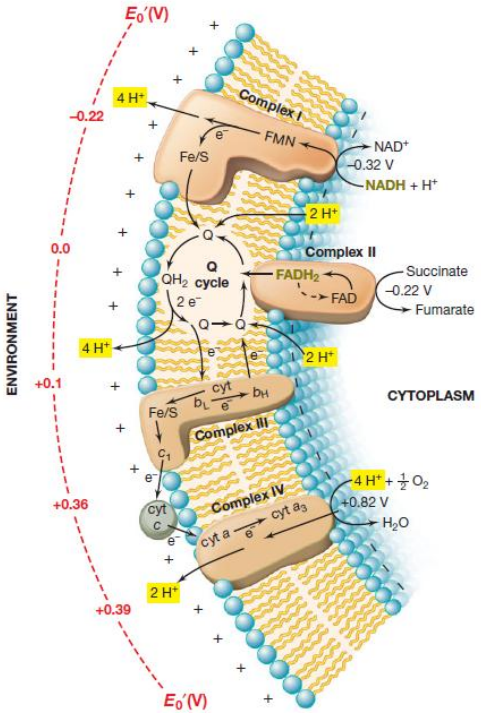

Figure 3.8 – Example of electron transport chain during aerobic respiration from *Paracoccus denitrificans*, a model organism for respiration studies, (Mandigan *et al.*, 2010).

The synteny study here shows an association in many species of *cybB* and BCN. This former gene encodes a putative cytochrome b561, a membrane protein with distinct molecular organization and spectral properties from other *b*-type cytochromes (Murakami *et al*., 1986). Its eukaryotic orthologous are functionality well described (Tsubaki *et al*., 2005; Lu *et al*., 2014), intervening on iron absorption (Glanfield *et al*., 2010) and in the glutathione-ascorbate cycle (Perin *et al*., 1988; Bérczi & Zimányi, 2014), regenerating vitamin E, which is responsible for processing ROS, by transferring electrons across the membrane from vitamin C (Lu *et al*., 2014). However, its functionality in prokaryote organisms remains unclear (Murakami *et al*., 1986). El-Halfawy & Valvano (unpublished) have shown that *cybB* is up-regulated by oxidative stress conditions. Purified CybB from *E. coli* is reduced by respiration substrates as a component of ETC, suggesting that these proteins intervene on ETC as electrons donors (Murakami *et al*., 1986). Further, the CybB redox potential was calculated as + 20 mV, depending on the cell physiological state, placing it before ubiquinones, a type of IQ (Kracke *et al*., 2015) in ETC. Together, these observations, coupled to the synteny data in this thesis, suggest that BCNs could play a significant role in ETC.

This hypothesis predicts that bacteria not requiring oxygen for respiration would not have BCNs. This is supported by the observation in this thesis that *E. faecium* lacks BCN orthologs. *E. faecium* is strict fermenter and it lacks ETC, Kreb's cycle and cytochromes (Willett, 1992). To strengthen our hypothesis that BCN and *cybB* might play a role on ETC and therefore be only present in organisms that are capable of aerobic respiration, we investigated other strict anaerobes. *Streptococcus pyogenes* (Gibson *et al*., 2000), *Clostridium* species and *Acetobacterium woodii* (Kracke *et al*., 2015), which lack ETC genes also lack BCN orthologs (Table 3.2). Together, the presence/absence of BCN and CybB supports the notion of these proteins being involved in ETC metabolism. Moreover, STRING database predictions suggest that these two genes are co-expressed, thus they might be expressed under the same physiological conditions, such as oxidative stress. In addition, microarray analyses suggest that BCN gene expression in *E. coli* is post-transcriptionally repressed by the FNR regulon overexpression (Durand & Storz, 2010). FNR consists on an oxygen sensor being active under anaerobic conditions. Its activation modulates the expression of a number of genes responsible for aerobic functions, repressing them and activating several genes encoding for anaerobic pathway (Salmon *et al*., 2003; Kang *et al*., 2005; Constantinidou *et al*., 2006) at transcriptional level, as well as post-transcriptional level through small RNAs (sRNA) by binding to mRNA and proteins (Waters & Storz, 2009). Also, studies made in *Neisseria meningitis* GNA1030 (Donnarumma *et al*., 2015) showed that specific inhibitors of IQ redox action are applied to WT and *Δgna1030* (*N. meningitis* BCNs) influence the survival of the bacteria, suggesting that BCNs intervene at some level on ETC.

Table 3.2 – Presence/absence of BCN and CybB in the genome of the indicated species based on BLASTp results. ETC presence/absence based on bibliography. *cybB* association with BCN gene obtained from synteny results. Among *Enterobacter* species only *Enterobacter* sp. and *Enterobacter cloacae* have *cybB* associated to BCN. NA = Not Applicable

Species	BCN	CybB	ETC	*cybB* associated of BCN gene
B. cenocepacia	+	+	+	+
E. coli	+	+	+	+
ESKAPE				
K. pneumoniae	+	+	+	-
A. baumanni	+	+	+	-
S. aureus	+	+	+	-
E. faecium	-	-	-	NA
P. aeruginosa	+	+	+	+
Enterobacter species	+	+	+	*Enterobacter* spp. and *E. cloacae*
Strict/aerotolerant anaerobes				
S. pyogenes	-	-	-	NA
Clostridium species	-	-	-	NA
A. woodii	-	-	-	NA

Here, I suggest a model of BCNs cellular function, where these proteins are capable to hijack and/or storage and/or transport hydrophobic compounds, such as vitamin E and IQ, to CybB where these compounds are reduced by electrons from substrates of ETC and/or vitamin C (Figure 3.9). The resulting reduced compounds, such as IQ is then transported into the bacterial membrane entering on the ETC. This hypothesized pathway would be unnecessary or inactive under anaerobic conditions.

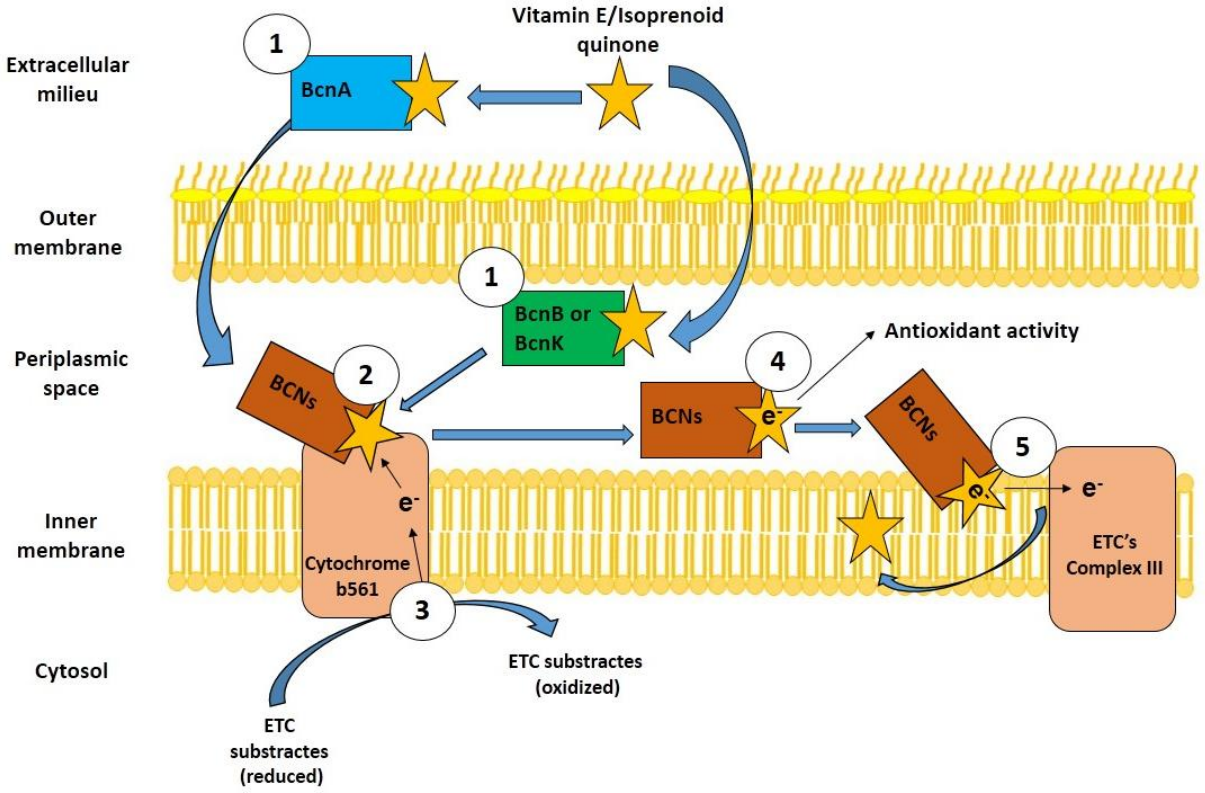

Figure 3.9 – Schematic model of BCN cellular function. **1** – Extracellular BcnA or BcnK/BcnB on the periplasmic space can bind hydrophobic compounds such as quinones or vitamin E. **2** – BCNs deliver these compounds to cytochrome b561 (CybB). **3** – ETC substrates are oxidized on the cytosol by CybB that transports electron across the membrane reducing the compound present on BCNs. **4** – the reduced compound is transported in the periplasmic space acting as an antioxidant or **5** – Reduced isoprenoid quinones are transported into the inner membrane where they enter in the ETC or act as antioxidants.

4. Conclusions and perspectives

Numerous attempts to purify a stable *K. pneumoniae*'s BCN exhibiting antibiotic binding failed. This was probably due to BCN membrane lipid anchor that resulted in aggregation when dialyzed in PBS. Also, protein overexpression on the cytosol, in *E. coli*, resulted on misfolded BcnK.

The construction of a *K. pneumoniae* BcnK defective mutants was attempted via unmarked deletion and insertion deletion methods. However, no mutants were obtained, thus *bcnK* essentiality was assessed by creating *bcnK* under the control of rhamnose inducible promoter. The *bcnK* rhamnose conditional mutants showed no lethal phenotype when grown in glucose, despite we showed that the rhamnose inducible promoter is functional in *K. pneumoniae*.

This may be due to a low level of constitutive read-through transcription of *bcnK*, which would lead to a low level of protein expression that might be sufficient to overcome lethality. Alternatively, *bcnK* may not be an essential gene for *K. pneumoniae*, but this is not supported by the deletion and insertion mutagenesis results that consistently failed despite many repetitions.

Preliminary results from oxidative stress assays suggest that *bcnK* is upregulated under oxidative stress conditions induced by paraquat. However, future assays using non-"regulated" gene by oxidative stress will be assessed as a negative control.

B. cenocepacia defective on BCNs production was complemented with *K. pneumoniae* BCN and assessed for antibiotic MIC restoration. The MIC is indeed restored, however, it appears to be due to the used vector and not BcnK itself. Future assays will be addressed by cloning *bcnK* into the same vector as was used for *B. cenocepacia* studies.

Structural modeling of BcnK in comparison to BcnA revealed two putative residues involved in antibiotic binding, Val107 and Glu118, which corresponded to BcnA Asp82 and Asp 93, respectively. However, the hydrophobicity characteristics of Asp and Glu residues are different and it remains unknown how these residues participate in antibiotic binding. Nonetheless, this information could be useful for future protein antibiotic docking models and prediction of important restudies for site directed mutagenesis.

Klebsiella spp. BCNs genomic comparisons indicated that these proteins are highly conserved despite they can be separated into three main clusters.

BCNs synteny and BLASTp studies showed that BCNs are associated with *cybB* and in some bacteria, while both genes are absent in all examined strict anaerobes. This information allows us to suggest a model of BCNs cellular function whereby BCN acts to capture or transporter hydrophobic compounds that will be reduced by CybB, thus contributing to antioxidant activities of bacteria under aerobic conditions.

CHAPTER IV - BIBLIOGRAFY

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CHAPTER V – APPENDIXES

5.1 – SUPPLEMENTARY TABLES

Table S1 – Strains, mutants and vectors used or created in this study. The following abbreviations stands for the respective antibiotic resistance: Tp^R: trimethoprim; Tet^R: tetracycline; Amp^R: ampicillin; Km^R: kanamycin; Em^R: erythromycin.

Strain or Plasmid	Description	Source/reference
Strains		
Escherichia coli		
DH5α	F^- φ80d*lacZ*ΔM15 (Δ*lacZYA-argF*)U169 *endA1 recA1 hsdR17*(r_K^- m_K^+) *supE44 deoR thi-1 nupG* Δ*gyrA96 relA1*, λ^-	Lab stock
GT115	F^- *mcrA* Δ(*mrr-hsdRMS-mcrBC*) φ80lacZΔM15 Δ*lacX74 recA1 rspL*(*StrA*) *endA1* Δ*dcm uidA*(Δ*MluI*)::pir-*116* Δ*sbcC-sbcD*	InvivoGen
BL21	F^- *dcm ompT hsdS* (r_B^- m_B^-) *gal*	Novagen
β2163	F^- RP4-2-Tc::Mu Δ*dapA*::(*erm-pir*) [Km^R Em^R]	Institute Pasteur
Klebsiella pneumoniae		
kp52.145	Clinical isolate (serotype O1:K2)	Nassif *et al*. (1989)
DNG1	kp52.145; P_{oxyR}::*luxCDABE*; Tp^R	This study
DNG6	kp52.145; P_{bcnK}::*luxCDABE*; Tp^R	This study
DNG15	kp52.145; P_{waaE}::*luxCDABE*; Tp^R	This study
DNG9	kp52.145; P_{rha}::*bcnK*; Tp^R	This study
DNG10	kp52.145; P_{rha}::*bcnK*; Tp^R	This study
DNG11	kp52.145; P_{rha}::*bcnK*; Tp^R	This study
DNG12	kp52.145; P_{rhaB2}-e-GFP	This study
Pseudomonas aeruginosa		
PAO1	Non-CF clinical isolate	Holloway (1955)
Burkholderia cenocepacia		
K56-2	ET12 clone related to J2315, clinical isolate	BCRRC
XOA10	K56-2; P_{rha}::*BCAL1928*; Tp^R	Ortega *et al*. (2007)
XOA12	K56-2; P_{rha}::*arnB*; Tp^R	Ortega *et al*. (2007)
OME4	K56-2; *ΔBcnAΔBcnB*	El-Halfawy & Valvano (2013)
DNG4	OME4; pDG1; Tet^R	This study
DNG5	OME4; pDA-CTHis; Tet^R	This study
Vectors		
pDA-CTHis	ori_{pBBR1}, *mob*$^+$ P_{dhfr}, C-terminal His-tag, Tet^R	D. Aubert, unpublished
pGPI-SceI-2	ori_{R6K} ΩTp^R *mob*$^+$, including an I-SceI restriction site; Tp^R	Flannagan *et al*. (2008)
pGPI-SceI-SacB	ori_{pBBR1} Tet^R *mob*$^+$, expressing I-SceI, SacB; Tet^R	Hamad *et al*. (2010)
pGSVTp-lux	ori_{R6K} Tp^R *luxCDABE*, derivate from pGSV3-Lux by replacing Gm^R for Tp^R cassette	Bernier *et al*. (2008)
pGEM®-T Easy	ori_{f1} *lacZ*; Amp^R	Promega
pET28a(+)	ITPG inducible plasmid, Km^R	Novagen
pSCRhaB2-e-GFP	ori_{pBBR1} *rhaR rhaS* P_{rhaB} *mob*$^+$; Tp^R	Cardona *et al*. (2005)
pGPΩTp	ori_{R6K} ΩTp^R cassete *mob*$^+$; Tp^R	Flannagan *et al*. (2007)
pSC200	ori_{R6K} *rhaR rhaS* P_{rhaB} *mob*$^+$; Tp^R	Ortega *et al*. (2007)
pUC19	ori_{pMBI}, *lacZα*; Amp^R	Invitrogen
pRF130	ori_{pBBR1}, P_{rhaB}, *mob*$^+$; Tp^R	Flannagan *et al*. (2007)
pDG1	pDA-CTHis, *bcnK*, C-terminus His-tag, Tet^R	This Study
pDG5	pDA-CTHis, *bcnK* without signal peptide, C-terminus His-tag, Tet^R	This Study
pDG7	pET28a(+), *bcnK* without signal peptide, N- and C-terminus His-tag, Km^R	This Study
pDG8	pET28a(+), *bcnK* without signal peptide, N-terminus His-tag, Km^R	This Study
pDG11	pUC19, *bcnK* without signal peptide, C-terminal His-tag, Amp^R	This study
pDG2	pGPI-SceI-2 containing fragments (≈1000 bp) flanking *bcnk*; Tp^R	This Study
pDG4	pGPI-SceI-2 containing fragments (≈300 bp) flanking *bcnK*; Tp^R	This Study
pDG9	pGPΩTp, 297 bp internal fragment from *bcnK*; Tp^R	This Study
pDG10	pSC200, 306 bp upstream region from *bcnK*; Tp^R	This Study
pDG3	pGSVTp-*lux* P_{oxyR}::*luxCDABE* Tp^R	This Study
pDG6	pGSVTp-*lux* P_{waaE}::*luxCDABE* Tp^R	This Study
pDG12	pGSVTp-lux P_{bcnK}::*luxCDABE*; Tp^R	This Study

Table S2 – Primes generated for this study and respective restriction enzymes. Underlined bases represent the restriction site. Bases in bold represents the homology region with pGSVTp-*lux* vector

Purpose	Primer no.	Oligonucleotide sequence, 5'-3'	Restriction site
Gene cloning	Q775	GGGCCCCATATGATGTTTTCAATAAGCCCTGTA	*NdeI*
	Q776	GGGCCCTCTAGATTTTGCATAGGCTTCCGTG	*XbaI*
	Q873	GGGTTTCATATGGCGGCTAAGGAATACAGTCTTG	*NdeI*
	Q880	GGGCCCCTCGAGTTTTGCATAGGCTTCCGTG	*XhoI*
	Q895	GGGCCCCTCGAGTTATTTTGCATAGGCTTCCGTG	*XhoI*
	Q841	**AAATAGCTCATACACCCAAACAGTA**GAATTCGGCCTACAACAAAGACATGC	*EcoRI*
	Q842	**GGCGGCACTTGTGATTAAGAGTCA**GAATTCTATTCATCCTCCATCGCCAC	*EcoRI*
	Q852	**GGCCGCACTTGTGTATAAGAGTCA**GAATTCACTATTTATCCGTTCTCTGTTTTGGCGGGC	*EcoRI*
	Q853	**AAATAGCTCATACACCCAAACAGTA**GAATTCCGGCACCTATGTGTACGAAAAGCGCC	*EcoRI*
	Q961	TTCCTTAGCCGCCATTGGATCCTCTAGAGTC	-
	Q962	GACTCTAGAGGATCCAATGGCGGCTAAGGAA	-
***K. pneumoniae* Mutagenesis**	Q786	CCCTTTTCTAGATAATTTCGTTGCGTAAACTT	*XbaI*
	Q787	CCCAAACTCGAGGTACAGAAAGCAATGCGGTT	*XhoI*
	Q788	CCCGGCTCGAGCGTTCAGACTTTAAGCTTGATAA	*XhoI*
	Q789	TTTGGGAATTCGAACCGACCGATCCTGACC	*EcoRI*
	Q810	CAACTCTTACACGATGAAGG	-
	Q811	TCTAGAAAACATTTGTTATTCCCTTTC	*XbaI*
	Q812	TCTAGAGCCTATGCAAAATAAGTAAA	*XbaI*
	Q813	GAATGACCAGCCAGTTCACC	-
	Q902	GGGCCCTCTAGACCGCATTGCTTTCTGTACTTGCC	*XbaI*
	Q903	GGGCCCGAATTCGTTGGGAAACGTTTTAAC	*EcoRI*
	Q904	GGGCCCTCTAGAGGTTTTCACTGGCAGCGTGA	*XbaI*

Table S3 – List of genomic and protein sequences obtained from NCBI used in this thesis with information referring species, strain, type of sequence (nucleotide or protein), accession number, description of the sequence and entry date.

Species	Strain	Type	Accession Number	Description	Entry Date
K. pneumoniae	kp52.145	Nucleotide	FO834906.1	Full genome sequence	09/08/2015
K. pneumoniae	kp52.146	Protein	CDO15049.1	YceI	09/08/2015
B. cenocepacia	J2315	Protein	CAR53634.1	BCAL3311	09/08/2015
B. cenocepacia	J2316	Protein	CAR53635.1	BCAL3312	02/08/2016

Table S4 – Composition of the solution used for protein purification protocols. (**A**) Lysis buffer; (**B**) Equilibration buffer; (**C**) Washing and elution buffers used for BcnK purification.

A

Lysis buffer (10 mL)	
Final concentration	**Volume**
20 mM Na_2HPO_4	2 mL of 0.1 M
500 mM NaCl	1 mL of 5 M
10 mM $MgCl_2$	0.2 mL of 0.5 M
DNAse 50 µg/mL	0.5 mg
Protease Inhibitor 1x	1 mL 10x
10 mM Imidazole	100 µl of 1M
10% Glycerol	1 mL
0.2% Triton X-100	20 µL
Milli-Q Water	4.68 mL

B

Equilibration buffer	
Final concentration	**Volume**
20 mM Na_2HPO_4	20 mL of 0.1 M
500 mM NaCl	10 mL of 5 M
10 mM Imidazole	1 mL of 1 M
Milli-Q water	69 mL

C

Purification buffers			
Final concentration	**50 mM Imidazole Washing buffer**	**75 mM Imidazole Washing Buffer**	**400 mM imidazole Elution Buffer**
20 mM Na_2HPO_4	10 mL of 0.1 M	10 mL of 0.1 M	3 mL of 0.1 M
1.5 mM NaCl	15 mL of 5 M	-	-
150 mM NaCl	-	1.5 mL of 5 M	450 µL of 5 M
Protease Inhibitor 1X	-	-	1.5 mL of 10x
10 mM Imidazole	2.5 mL of 1 M	3.75 mL of 1 M	6 mL of 1 M
Milli-Q Water	22.5 mL	34.75 mL	4.05 mL
Final volume	50 mL	50 mL	15 mL

Table S5 – List of bacterial strains possessing a BCNs homologous obtained from BLASTp results, deploying BcnK as query and used to construct the cladogram from Figure S3. The species code, strains, accession number and entry date are associated with the attributed cluster (**1**, **2** or **3**). Similar protein sequences are grouped in MULTISPECIES (more than one species) or MULTISTRAINS (more than one strain). *Klebsiella pneumoniae* (Kp), *Klebsiella oxytoca* (Ko), *Klebsiella variicola* (Kv), *Klebsiella quasipneumoniae* (Kqp).

Species	Strain	Accession Number	Entry Date	Cluster
Kp	kp52.145	CDO15049.1	02/08/2016	**1**
Kp	BWH 46	KDH45308.1	02/08/2016	**1**
Kp	B5055	WP_016528802.1	02/08/2016	**1**
Kp	IS43	CDL09552.1	02/08/2016	**1**
Kp; Ko	MULTISPECIES (510 results)	WP_004148751.1	02/08/2016	**1**
Kp; Ko	MULTISPECIES (47 results)	ESM52217.1	02/08/2016	**1**
Kp	k1773	SAV78042.1	02/08/2016	**1**
Kp	MULTISTRAINS (13 results)	WP_021313024.1	02/08/2016	**1**
Kp	BIDMC 36	ESL39202.1	02/08/2016	**1**
Kp	UCI76	KMI59035.1	02/08/2016	**1**
Kp	k263	SAT12336.1	02/08/2016	**1**
Kp	MGH 66	KDL58125.1	02/08/2016	**1**
Kp	W2-1-ERG2	SBY54108.1	02/08/2016	**1**
Kp	MS 92-3	EGF61318.1	02/08/2016	**1**
Kp	k2334	SAV41132.1	02/08/2016	**1**
Kp	UHKPC81	EOY77384.1	02/08/2016	**1**
Kp	UCI 26	EWD54348.1	02/08/2016	**1**
Kp	BIDMC 25	ESL49963.1	02/08/2016	**1**
Kp	ATCC 13884	EEW41939.1	02/08/2016	**1**
Kp	BIDMC 47	EWE15600.1	02/08/2016	**1**
Kp	UCI70	KMI53420.1	02/08/2016	**1**
Kp	k2290	SBI21147.1	02/08/2016	**1**
Kp	NTUH-K2044	BAH63914.1	02/08/2016	**1**
Kp	k722	SAX24660.1	02/08/2016	**1**
Kp	UCI94	KMI90325.1	02/08/2016	**1**
Kp	98_KPNE	WP_049204778.1	02/08/2016	**1**
Kp	MULTISTRAINS (206 results)	WP_004151852.1	02/08/2016	**1**
Kp	MULTISTRAINS (35 results)	WP_004891166.1	02/08/2016	**1**
Kp	k1773	WP_064152600.1	02/08/2016	**1**
Kp	6234	KHQ25135.1	02/08/2016	**1**
Kp	k1781	SAY06168.1	02/08/2016	**1**
Kp	k1319	SAW93657.1	02/08/2016	**1**
Kp	SB3193	CDQ55550.1	02/08/2016	**1**
Kp	CHS162	KMD23806.1	02/08/2016	**1**
Kp	F1-9-ERG1	SBZ89122.1	02/08/2016	**1**
Kp	CHS136	KMX50881.1	02/08/2016	**1**
Kp	k414	SAT33337.1	02/08/2016	**1**

Kp	MGH116	KMH67375.1	02/08/2016	**1**
Kp	SKLX2993	WP_040225745.1	02/08/2016	**1**
Kp	KP-11U	WP_032412495.1	02/08/2016	**1**
Kp	MULTISTRAINS (40 results)	WP_004898943.1	02/08/2016	**1**
Kp	k263	WP_064161572.1	02/08/2016	**1**
Kp	KP-1	WP_032628321.1	02/08/2016	**1**
Kp	ST323:941530379	WP_040148358.1	02/08/2016	**1**
Kp	SKLX2891	WP_065890525.1	02/08/2016	**1**
Kp	SB3432	WP_020947796.1	02/08/2016	**1**
Kp	50531633	WP_032429905.1	02/08/2016	**1**
Kp	MULTISTRAINS (4 results)	WP_032438274.1	02/08/2016	**1**
Kp	MULTISTRAINS (2 results)	WP_032415997.1	02/08/2016	**1**
Kp	MULTISTRAINS (2 results)	WP_064172264.1	02/08/2016	**1**
Kp	MULTISTRAINS (4 results)	WP_032420194.1	02/08/2016	**1**
Kp	k2290	WP_064146264.1	02/08/2016	**1**
Kp	DHQP1002001	WP_068814988.1	02/08/2016	**1**
Kp	yzusk-4	AKG99567.1	02/08/2016	**1**
Kp	UCI70	WP_048333775.1	02/08/2016	**1**
Kp	k722	WP_064181430.1	02/08/2016	**1**
Kp	5422	WP_031593102.1	02/08/2016	**1**
Kp	MULTISTRAINS (3 results)	WP_016831241.1	02/08/2016	**1**
Kp	CCGT01000026	WP_040188956.1	02/08/2016	**1**
Kp	359_ECLO	WP_048969219.1	02/08/2016	**1**
Kp	F1-9-ERG1	WP_065808059.1	02/08/2016	**1**
Kp	CHS162	WP_048290620.1	02/08/2016	**1**
Kp	CHS136	WP_050885681.1	02/08/2016	**1**
Kp	570_KPNE	WP_048987887.1	02/08/2016	**1**
Kp	MULTISTRAINS (3 results)	WP_046623832.1	02/08/2016	**1**
Kp	MSULTISTRAINS (6 results)	WP_038431357.1	02/08/2016	**1**
Kp	KLP28	KTG51533.1	02/08/2016	**1**
Kp	K57-33	WP_060528076.1	02/08/2016	**1**
Kp	CFSAN044574	WP_069345197.1	02/08/2016	**1**
Kp; Kv	MULTISPECIES (19 results)	WP_008804418.1	02/08/2016	**2**
Kp	YH43	WP_061154214.1	02/08/2016	**2**
Kqp	21_GR_13	WP_065881285.1	02/08/2016	**3**
Kp	MULTISTRAINS (4 results)	WP_023297838.1	02/08/2016	**2**
Kp; Kqp; Kv	MULTISPECIES (21 results)	WP_004203155.1	02/08/2016	**3**
Kp	MGH 80	WP_032735938.1	02/08/2016	**2**
Kp	50878013	WP_060619667.1	02/08/2016	**2**
Kp	837_KPNE	WP_049010347.1	02/08/2016	**2**
Kp	MGH92	WP_048330424.1	02/08/2016	**2**
Kp; Kv	MULTISPECIES (12 results)	WP_016161315.1	02/08/2016	**2**
Kp; Kv	MULTISPECIES (2 results)	WP_064323602.1	02/08/2016	**2**
Kv	801	WP_043875107.1	02/08/2016	**2**
Kv	At-22	WP_012967822.1	02/08/2016	**2**
Kp	k2254	WP_064154439.1	02/08/2016	**3**

Kqp	KPSB59	WP_032453272.1	02/08/2016	**3**
Kqp	07A044	WP_032456269.1	02/08/2016	**3**
Kqp	FI_HV_2014	WP_050533298.1	02/08/2016	**3**
Kqp	385_ECLO	WP_049116548.1	02/08/2016	**3**
Kp	12 3578	WP_017900775.1	02/08/2016	**3**
Kp	Multistrains (3 results)	WP_023318555.1	02/08/2016	**3**
Kp	UCICRE 14	WP_032428892.1	02/08/2016	**3**
Kqp	865_KPNE	WP_049013562.1	02/08/2016	**3**
Kqp	KQQSB11	WP_044523846.1	02/08/2016	**3**
Kp	k2254	SAU45131.1	02/08/2016	**3**
Kqp	01A030	CDQ14977.1	02/08/2016	**3**
Kp	k1457	SAX28671.1	02/08/2016	**3**
Kqp	18A069	CDN00725.1	02/08/2016	**3**
Kp	W2-15-ERG14	SCA20417.1	02/08/2016	**3**
Kp	MGH96	KMH14419.1	02/08/2016	**3**
Kp	UCICRE 14	ESL74126.1	02/08/2016	**3**
Kp	MGH113	KMH48341.1	02/08/2016	**3**

5.2 – SUPPLEMENTARY FIGURES

MFSISPVFFWANIYVPADFEDYCVNTLKKTALLSVLALYIPVSQAAAKEYSLDPQHTSVVISWNHFGFSNPTAYISDVSG
KLAFDKENPEKSSVNVTLPVKTIDAHVKALTDEFLGKEYFDVKTFPNATFQSTKVESKGDNKYDVEGNLTIKGITKPVVLH
AVLNKQDMHPMVKKEAIGFDATGVIKRSDFKLDKYVSAVSDNVTITLSTEAYAK

Figure S1 – BcnK amino acid sequence displaying its putative signal peptide (green), predicted to be cleaved between Alanine45 and Alanine46 (SQA-AA).

Figure S2 – Constructed vector pDG11 showing *lacZα* gene fragment and N-region of *bcnK* coding region with the respective primers for point mutation assays. The primers display the adenine to be added aiming to fuse both genes.

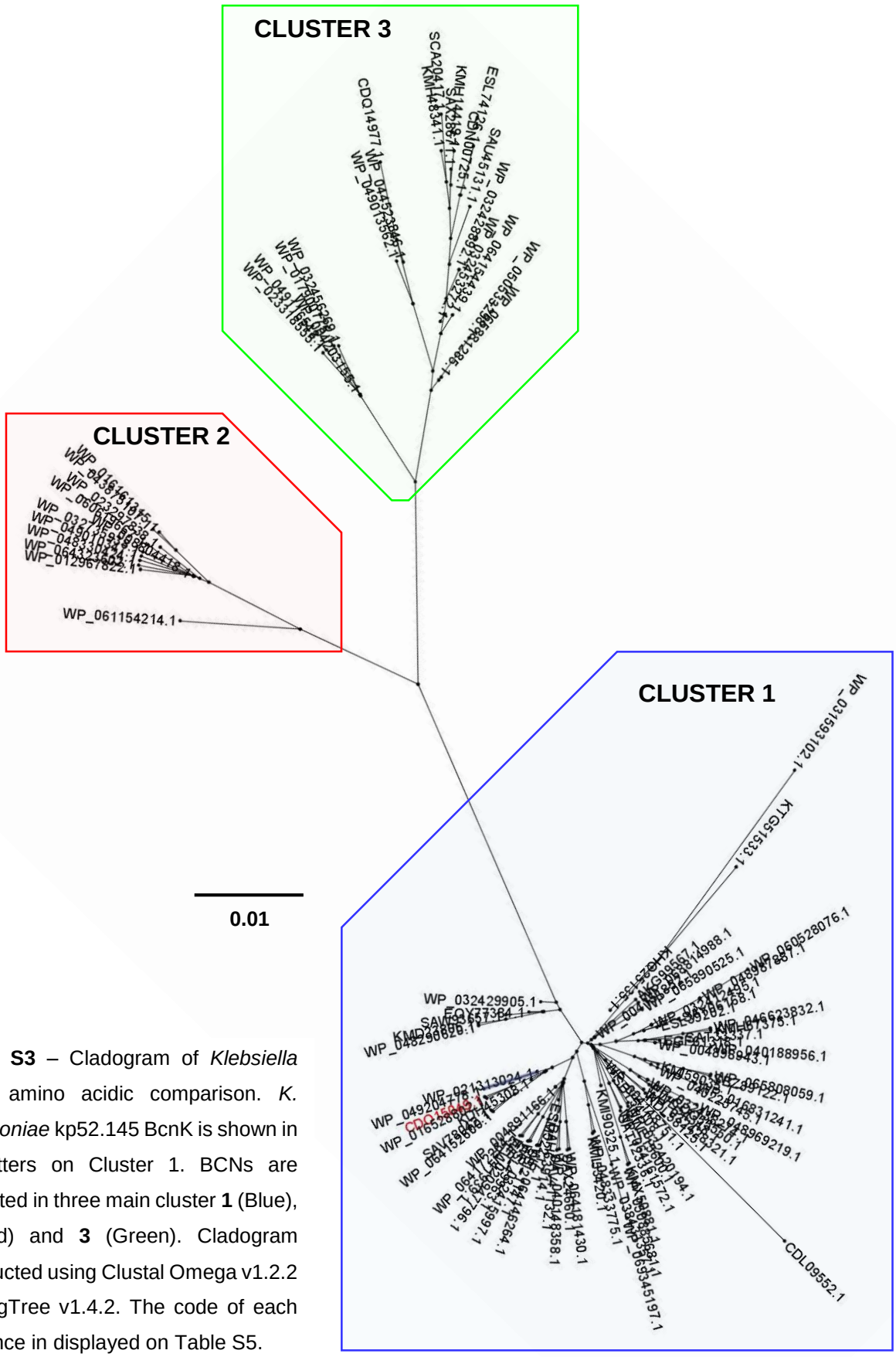
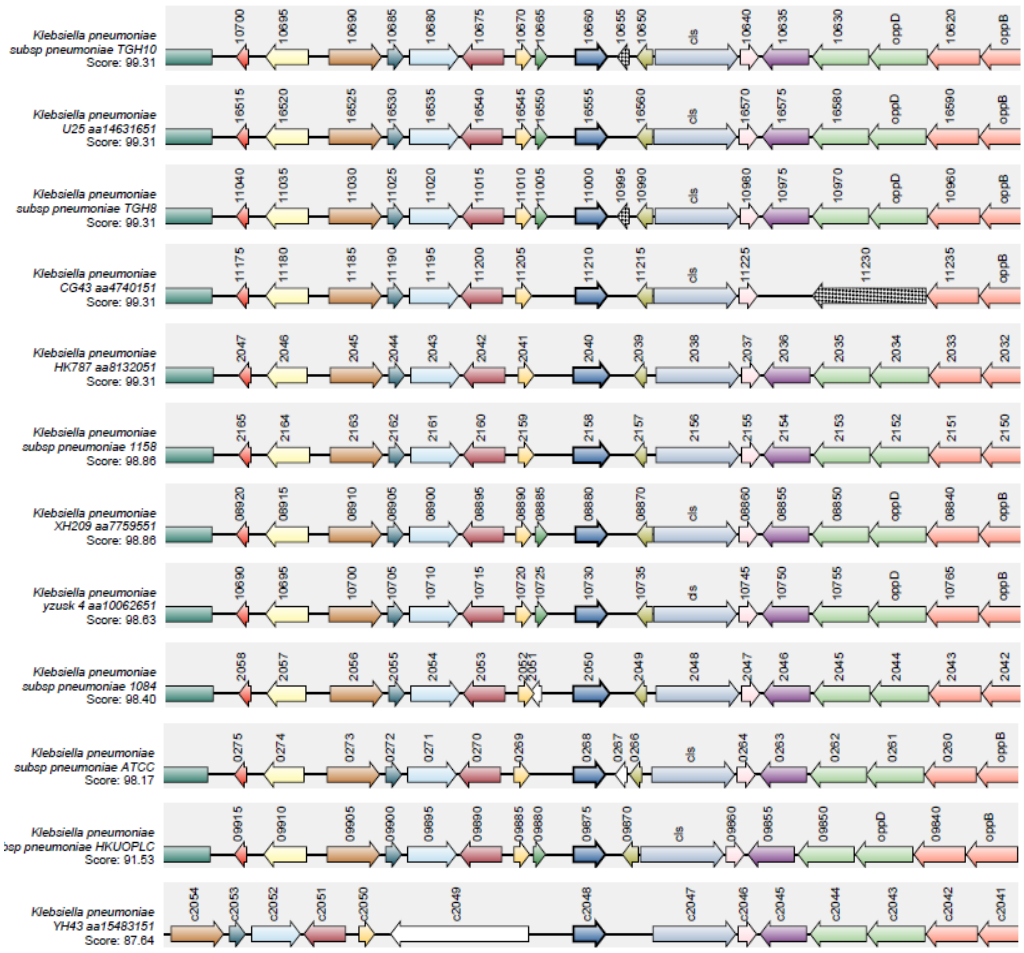


Figure S3 – Cladogram of *Klebsiella* genus amino acidic comparison. *K. pneumoniae* kp52.145 BcnK is shown in red letters on Cluster 1. BCNs are separated in three main cluster **1** (Blue), **2** (Red) and **3** (Green). Cladogram constructed using Clustal Omega v1.2.2 and FigTree v1.4.2. The code of each sequence in displayed on Table S5.

Genomic contexts



SyntTax Report http://archaea.u-psud.fr/SyntTax

Query protein sequence:

```
mfsispvffwaniyvpadfedycvntlkktallsvlalyipvsqaaakeysldpqhtsvviswnhfgfsn
ptayisdvsgklafdkenpekssvnvtlpvktidahvkaltdeflgkeyfdvktfpnatfqstkveskgd
nkydvegnltikgitkpvvlhavlnkqdmhpmvkkeaigfdatgvikrsdfkldkyvsavsdnvtitlst
eayak
```

Genomes without synteny:

```
Klebsiella_pneumoniae_30660_NJST258_1_aa5980051
Klebsiella_pneumoniae_30684_NJST258_2_aa5979051
Klebsiella_pneumoniae_32192_aa8073953
Klebsiella_pneumoniae_342_aa195651
Klebsiella_pneumoniae_34618_aa8143051
Klebsiella_pneumoniae_500_1420_aa4067652
Klebsiella_pneumoniae_ATCC_BAA_2146_aa3643853
Klebsiella_pneumoniae_blaNDM_1_aa7354951
Klebsiella_pneumoniae_CAV1193_aa14561351
Klebsiella_pneumoniae_CAV1344_aa10221751
Klebsiella_pneumoniae_CAV1392_aa10220351
Klebsiella_pneumoniae_CAV1596_aa10222351
Klebsiella_pneumoniae_DMC1097_aa4172252
Klebsiella_pneumoniae_J1_aa14823451
Klebsiella_pneumoniae_JM45_aa4454051
Klebsiella_pneumoniae_KCTC_2242_aa2204851
Klebsiella_pneumoniae_KP_1_aa4659752
Klebsiella_pneumoniae_Kp52_145_aa9681551
Klebsiella_pneumoniae_KP617_aaI3071751
Klebsiella_pneumoniae_KpN01_aa14560553
Klebsiella_pneumoniae_MS6671_aa14559951
Klebsiella_pneumoniae_PMK1_aa7646151
Klebsiella_pneumoniae_RYC492_aa3461451
Klebsiella_pneumoniae_subsp_pneumoniae_234_12_aa9810451
Klebsiella_pneumoniae_subsp_pneumoniae_HS11286_aa2401852
Klebsiella_pneumoniae_subsp_pneumoniae_Kp13_aa5121651
Klebsiella_pneumoniae_subsp_pneumoniae_KP5_1_aa7146351
Klebsiella_pneumoniae_subsp_pneumoniae_KPNIH1_aa2815352
Klebsiella_pneumoniae_subsp_pneumoniae_KPNIH10_aa2814352
Klebsiella_pneumoniae_subsp_pneumoniae_KPNIH24_aa7146751
Klebsiella_pneumoniae_subsp_pneumoniae_KPNIH27_aa6959351
Klebsiella_pneumoniae_subsp_pneumoniae_KPNIH29_aa7849451
Klebsiella_pneumoniae_subsp_pneumoniae_KPNIH30_aa7849851
Klebsiella_pneumoniae_subsp_pneumoniae_KPNIH31_aa7850051
Klebsiella_pneumoniae_subsp_pneumoniae_KPNIH32_aa7753951
Klebsiella_pneumoniae_subsp_pneumoniae_KPNIH33_aa7753751
Klebsiella_pneumoniae_subsp_pneumoniae_KPR0928_aa7175151
Klebsiella_pneumoniae_subsp_pneumoniae_MGH_78578_ATCC_700721_aa163051
Klebsiella_pneumoniae_subsp_pneumoniae_NTUH_K2044_aa98851
Klebsiella_pneumoniae_subsp_pneumoniae_NUHL24835_aa15218951
Klebsiella_pneumoniae_subsp_pneumoniae_PittNDM01_aa7332551
Klebsiella_pneumoniae_subsp_pneumoniae_RJF293_aaI5300151
Klebsiella_pneumoniae_subsp_pneumoniae_RJF999_aa15299351
Klebsiella_pneumoniae_subsp_rhinoscleromatis_SB3432_aa9678451
Klebsiella_pneumoniae_UHKPC07_aa4172652
Klebsiella_pneumoniae_UHKPC33_aa4170852
```

Figure S4 – SyntTax report obtain for BcnK. At the center, in blue and bold, stands *bcnK*, each arrow represents conserved a gene with an associated color. Genes with no synteny are presented in white genes.